Non-invasive extracellular recordings of electrical activity from whole intact islets of Langerhans and the development of the medium throughput BetaScreen device

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„Geh aufrecht wie die Bäume. Lebe dein Leben so stark wie die Berge. Sei sanft wie der Frühlingswind und bewahre die Wärme der Sonne im Herzen."
(Weisheit der Navajo)

Meiner lieben Oma Frieda Schönecker gewidmet

Content

Abbreviations

$[Ca^{2+}]_c$	Intracellular Ca^{2+} concentration
$([Ca^{2+}]_m)$	mitochondrial Ca^{2+} concentration
ADP	Adenosine diphosphate
APs	Action potentials
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
Cat	Catalase
Ca_v channels	Voltage-gated Ca^{2+} channels
CHI	Congenital hyperinsulinism
EC_{50}	Half maximal effective concentration
FADH	Flavin adenine dinucleotide
FOOP	Fraction of Plateau Phase
GCK	Glucokinase
Gpx	Glutathione peroxidase
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
Glut-1	Glucose transporter 1
Glut-2	Glucose transporter 2
H^+	Hydrogen
H_2O_2	Hydrogen peroxide
IC_{50}	Half maximal inhibitory concentration
K_{ATP} channels	ATP-sensitive potassium channels
K_{Ca} channels	Ca^{2+}-activated K^+ channels
K_{IR} channels	Inwardly rectifying potassium channel
K_m	Michaelis constant
K_v channels	Voltage-dependent K^+ channels

MEA	Microelectrode Array
Na^+	Sodium ion
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
O_2^-	Superoxide anion radical
pMEA	perforated MEA
ROS	Reactive oxygen species
SSC	Stimulus-secretion coupling
SOD	Superoxide dismutase
SUR	Sulfonylurea receptors
T2DM	Type-2 diabetes mellitus
TEA	Tetraethylammonium
TTX	Tetrodotoxin
V_m	Membrane potential
$\Delta\Psi$	Mitochondrial membrane potential

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Zusammenfassung

Typ-2-Diabetes mellitus (T2DM) ist die häufigste und gleichzeitig folgen-schwerste Stoffwechselerkrankung weltweit. Neue Strategien zur Behandlung von T2DM und den damit verbundenen schwerwiegenden Folgeerkrankungen sowie die Entwicklung einer optimalen Medikation für alle Patienten sind daher sowohl in der akademischen Forschung, als auch in der pharmazeutischen Industrie eine wichtige Thematik. Die vorliegende Promotionsarbeit zeigt die Etablierung der Mikroelektrodenarray (MEA)-Technologie als neue und innovative Methode für die funktionale Untersuchung der in der Beta-Zelle entstehenden Veränderungen während der Entstehung von T2DM. Pfeiffer et al konnte erstmalig zeigen, dass die elektrische Aktivität von isolierten intakten Langerhans-Inseln reproduzierbar auf planaren Mikroelektroden extrazellulär gemessen werden kann (Pfeiffer et al., 2011). Die Ergebnisse der Messungen mit der MEA-Technologie sind qualitativ vergleichbar mit den Ergebnissen, die mit den klassischen elektrophysiologischen Techniken, wie zum Beispiel der patch-clamp Technik oder der intrazellulären Mikroelektrodentechnik gemessen wurden (Pfeiffer et al., 2011). Ferner habe ich zum ersten Mal eine Langerhans-Insel-Kultur auf Mikroelektroden entwickelt, mit der die Langerhans-Inseln *ex vivo* über einen Zeitraum von über einem Monat kultiviert werden konnten. Über die gesamte Zeit konnte die elektrische Aktivität stabil aufrechterhalten werden (Schönecker et al., 2014). Pathophysiologische Parameter, die die Entstehung von Diabetes begünstigen bzw. den Krankheitsverlauf beschleunigen, wie bspw. Überernährung und Bewegungsmangel, schädigen die Langerhans-Inseln durch eine vermehrte Generierung von u.a. reaktiven Sauerstoffspezies (ROS). Ich konnte durch Applikation von Wasserstoffperoxid (H_2O_2) oxidativen Stress auslösen, der einen signifikanten Effekt auf die elektrische Aktivität der Beta-Zellen hatte und der durch die Applikation von dem Antioxidans Tempol aufgehoben werden konnte. Die Ergebnisse dieser Studie belegen, dass die Gabe von antioxidativ wirkenden Substanzen, den verheerenden, durch ROS-ausgelösten Dysfunktionen entgegen wirken kann (Schönecker et al., 2014). In einem weiteren Teilprojekt der vorliegenden Arbeit konnte ich erstmalig elektrophysiologische Messungen mit humanen Langerhans-Inseln auf MEAs durchführen (Schönecker et al., 2015). Die durch das BMBF-Programm KMU Innovativ (Biotechnolgie-BioChance, #0316162B) geförderte Entwicklung des auf der MEA-Technologie basierenden BetaScreen-

Geräts ermöglicht erstmals parallelisierte, semiautomatische Messungen von intakten Langerhans-Inseln. Das gegenwärtige Layout ermöglicht simultane Messungen von bis zu 25 Langerhans-Inseln, was den Durchsatz der akuten Messungen um den Faktor 25 erhöht. Der Einsatz des BetaScreen-Geräts ist nicht nur auf murine Langerhans-Inseln limitiert, sondern kann auch für die Messung elektrischer Aktivität humaner Langerhans-Inseln verwendet werden. Somit konnte die MEA-Technologie als neue und innovative Methode für die Untersuchung von humanen und murinen Langerhans-Inseln etabliert werden.

Summary

Type-2 diabetes mellitus (T2DM) is a tremendous health problem worldwide. The present work demonstrates that the microelectrode array (MEA) technique is an excellent tool to study *in vitro* the molecular basis of functional changes in beta-cells during the development of T2DM. I have provided the "proof-of-principle" that electrical activity can be reproducibly recorded using intact single islets and extracellular electrode arrays. The results revealed with the MEA technology are comparable to results obtained with traditional electrophysiological techniques, like e.g. the patch clamp technique or recordings with intracellular electrodes (Pfeiffer et al., 2011). Moreover, I established an *in vitro* model that allows for the first time long-term investigations of beta-cell function through repetitive electro-physiological recordings of the same culture using the MEA technology (Schönecker et al., 2014). Further, I have shown that the application of oxidative stress which is crucial in the development of T2DM has a strong effect on the electrical behaviour of the single islets *in vitro* and that a SOD mimetic protects beta-cells against acute oxidative stress demonstrating the central role of SOD as an antioxidant defence mechanism within pancreatic islets (Schönecker et al., 2014). The next fundamental achievement was to show for the first time that the MEA technique allows measurements of electrical activity of islets isolated from human biopsies. Human islets exhibit glucose-dependent electrical activity and the electrical activity was increased by tolbutamide and inhibited by diazoxide. The Na^+ channel inhibitor tetrodotoxin markedly reduced electrical activity in human islets, but does not affect electrical activity of mouse islets (Schönecker et al., 2015). Within the scope of the BMBF-program KMU innovative (Biotechnol-gie-BioChance, #0316162B) and in cooperation with the company Multi Channel Systems (MCS) this study presents for the first time the development of the Be-taScreen device which allows to record semi automatically from up to 25 islets simultaneously. Importantly, the throughput of the acute recordings is now increased by a factor of 25 and paves the way for pharmaceutical medium-through-put drug screenings. Furthermore, I could show that the BetaScreen device is also suitable to record electrical activity from human islets.

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Pfeiffer, T., Kraushaar, U., Düfer, M., Schönecker, S., Haspel, D., Günther, E., Drews, G., and Krippeit-Drews, P. (2011). **Rapid functional evaluation of beta-cells by extracellular recording of membrane potential oscillations with microelectrode arrays.** Pflüg. Arch. Eur. J. Physiol. *462*, 835–840.

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Abstracts

Schönecker S., Pfeiffer T., Düfer M., Guenther E., Drews G., Krippeit-Drews P., Kraushaar U. Oscillatory activity in murine islets of Langerhans evaluated by extracellular recordings. The 90th Annual Meeting of The German Physiological Society, Regensburg, Germany, Acta Physiologica (2011)

Sven Schönecker, Martina Düfer, Peter Krippeit-Drews, Gisela Drews, Elke Guenther, Udo Kraushaar **Oscillatory activity in murine islets of Langerhans evaluated by extracellular recordings.** 8th Int. Meeting on Substrate-Integrated Microelectrode Arrays (2012)

Schönecker S, Kraushaar U, Guenther E, Düfer M, Sahr A, Härdtner C, Walther R, Lendeckel U, Barthlen W, Krippeit-Drews P, Drews G **Microelectrode array (MEA) technology allows functional analysis of human and mouse pancreatic islets**. Abstract of the 49th Annual Meeting of the EASD, Diabetologia (2013)

Sven Schönecker, Peter Krippeit-Drews, Gisela Drews, Karl-Heinz Boven, Elke Guenther, Udo Kraushaar **MEA technology – a New Player in Diabetes Research.** 8th Int. Meeting on Substrate-Integrated Microelectrode Arrays (2014)

Personal Contribution

The goal of my PhD study was to establish the MEA-technology as a new tool for the investigation of whole intact islets of Langerhans and to find a new strategy to protect the islets against diabetes inducing stress factors. I could show for the first time that oscillatory activity can be reproducibly recorded via the MEA technology. Moreover, I developed and established for the first time an *in vitro* model that allows long-term investigations of beta-cell physiology on top of MEA electrodes. Another fundamental achievement was that I was the first who could record electrical activity of isolated human islets on extracellular electrodes. I was responsible for the biological part of the development of the BetaScreen device which includes all test recordings with primary cells and improvements of the recordings assay. The data analysis and scientific interpretation of all electrical signals recorded from islets of Langerhans throughout the entire PhD study was carried out in agreement with my supervisors.

1   General Introduction

The reason why islets of Langerhans obtain a remarkably amount of consideration is due to their ability to produce and secrete the body's only hormone which is able to lower blood glucose concentration. The impairment of a proper insulin secretion can result in several devastating diseases, e.g. diabetes mellitus or congenital hyperinsulinism (CHI). Furthermore it is a matter of fact that especially Type-2 diabetes mellitus (T2DM), the most abundant endocrine disease, is an increasing health problem worldwide which has reached epidemic levels. The growing prevalence underlines the importance to further understand the physiology and pathophysiology of these fascinating islets.

1.1   Islets of Langerhans

The pancreas is an unpaired glandular organ which combines two main functions 1) a digestive function and 2) an endocrine function. The digestive function comprise the secretion of pancreatic juice containing digestive enzymes and bicarbonate directly into the small intestine. These digestive enzymes are responsible for the breakdown of lipids, proteins and carbohydrates. The endocrine function is to secrete important hormones, including insulin, glucagon, somatostatin, and pancreatic polypeptide into the blood stream. The islets of Langerhans, small micro-organs that consist of different cell types, are responsible for the secretion of these hormons. The islets of Langerhans lie scattered throughout the pancreas.

1.1.1   Anatomy of the islets of Langerhans

The anatomical nomenclature was established by Edouard Laguesse (1861-1927), the first who found out that they are involved in endocrine secretion. He called them islets of Langerhans after Paul Langerhans, who was the first descriptor of these micro-organs (Langerhans 1869). The average diameter of a human islet is 140 µm (Hellman, Hellerström, 1969). They are highly vascularised and are surrounded by a collagen capsule (Hughes et al., 2006). The human pancreas contains approximately 1 million islets (Hellman, 1959) which is about 1 to 2 % of the pancreatic cell mass. The islets comprise several different cell types, the most common ones are the insulin secreting beta-cells (up to 50 % in humans islets), glucagon producing alpha-cells (35-40 %), and the somatostatin

secreting delta-cells (10-15 %) (Cabrera et al., 2006). The secreted hormones leave the islets via small capillaries into the portal vein.

1.2   The physiological function of ion channels in beta-cells

Electrical excitability of beta-cells is strictly regulated by specific ion channels located in the plasma membrane. The interplay between glucose metabolism and ion channels in beta-cells leads to this unique characteristic oscillatory pattern of the membrane potential (V_m) (Drews et al., 2015).

1.2.1   ATP-sensitive potassium channels (K_{ATP} channels)

K_{ATP} channels are potassium channels regulated by the intracellular nucleotides ATP and ADP. They are hetero-octamers containing sulfonylurea receptors (SUR1, SUR2A and B) and inwardly rectifying potassium channels (K_{IR}) ($K_{IR}6.1$ or $K_{IR}6.2$) (Aguilar-Bryan et al., 1998; Babenko et al., 1998; Bryan et al., 2007; Clement et al., 1997; Inagaki et al., 1995). K_{ATP} channels of beta-cells are containing four $K_{IR}6.2$ pore-forming subunits and four regulatory SUR1 subunits (Aguilar-Bryan, 1999; Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997). The SUR1 subunit contains three transmembrane helices and two intracellular nucleotide-binding domains (Aguilar-Bryan et al., 1995). Interactions of the SUR1 subunit and MgADP lead to an increased open probability of the channel. The inhibitory effect of ATP is mediated through interactions with the pore-forming $K_{IR}6.2$ subunits (Tanabe et al., 1999, 2000; Tucker et al., 1997).

Drugs with an effect on K_{ATP} channel activity are often used to treat severe diseases like e.g. T2DM (Cook, 1995; Füchtenbusch et al., 2000) or CHI (Lindley and Dunne, 2005). Important antagonists are sulfonylureas and glinides, which were often used to improve insulin secretion in T2DM. The blockage of K_{ATP} channels leads to a glucose-independent depolarisation of V_m. Channel agonists like e.g. diazoxide hyperpolarise beta-cell V_m and are important for the treatment of insulinoma (Gill et al., 1997) or diazoxide-sensitive CHI (Lindley and Dunne, 2005).

1.2.2   Voltage-dependent Ca^{2+} channels (Ca_v channels)

Voltage-dependent Ca^{2+} channels (Ca_v) play an important role in glucose-induced insulin secretion by triggering the exocytosis of insulin containing vesicles.

Ca_v channels consist of a pore-forming α1 subunit with a voltage sensor, a selectivity filter for Ca^{2+} ions and so called activation and inactivation gates (Catterall, 2000; Yang and Berggren, 2006). The additional subunits ß, γ, and α2/δ are responsible for the regulation of activation and inactivation of the channel.

There are various types of Ca_v channels in beta-cells and the composition is species dependent. But for insulin secretion the L-type Ca^{2+} channel is considered to be the most important one. In rodent beta-cells almost the complete Ca^{2+} current is blocked by a specific L-type Ca^{2+} channel blocker (Gilon et al., 1997; Plant, 1988). In addition, also insulin secretion is nearly completely diminished by the blockage of L-type Ca^{2+} channels in rodent, as well as in human beta-cells (Braun et al., 2008; Satin et al., 1995).

As soon as the threshold for the activation of Ca_v channels is reached an influx of Ca^{2+} ions leads to an increase of $[Ca^{2+}]_c$ which directly triggers the exocytosis of insulin. Bursts of action potentials lead to an influx of Ca^{2+} ions with its repetitious pattern (oscillations) in the presence of a stimulatory glucose concentration (Bergsten et al., 1994; Gilon et al., 1993; Santos et al., 1991).

1.2.3 Ca^{2+}-activated K$^+$ (K_{Ca}) channels and voltage-dependent K$^+$ (K_v) channels

K_{ATP} and Ca_v channels are important for the initiation of electrical activity and insulin secretion. The main function of voltage-dependent K$^+$ (K_v) channels is the repolarization of action potentials (APs), whereas Ca^{2+}-activated K$^+$ (K_{Ca}) channels are involved in the regulation of V_m oscillations (Braun et al., 2008; Drews, 2009; MacDonald et al., 2002). That means, without the repolarizing properties of these channels, it would be impossible for beta-cells to oscillate. K_v channels consist of alpha subunits which form the conductance pore. Sequence homology studies of the alpha subunits revealed the existence of 12 different classes of K_v channels (Gutman et al., 2005). Five subfamilies (K_v1, 2, 3, 6 and 9) are already confirmed in primary beta-cells (Göpel et al., 2000; Jacobson and Philipson, 2007; MacDonald and Wheeler, 2003; Philipson et al., 1991; Roe et al., 1996; Yan et al., 2004). It could be shown that K_v currents consist of one 4-aminopyridine-insensitive current without inactivation which can be blocked by TEA$^+$ and one inactivating current which can be blocked by 4-aminopyridine (Düfer et al., 2004; MacDonald et al., 2001; Smith et al., 1989; Su et al., 2001).

There are three groups of K_{Ca} channels 1) large-conductance BK channels, 2) intermediate-conductance SK4 channels and 3) small-conductance K_{Ca} channels (SK1, SK2 and SK3) (Tamarina et al., 2003). In 1984 Cook and Hales found out how the closure of the K_{ATP} channels triggers the initiation of the APs (Ashcroft et al., 1984; Cook and Hales, 1984). However, the origin of the current which hyperpolarises V_m during the initiation of the interburst phase was still unknown. Göpel and colleagues where the first who described the so called slowly rising outward current (I_{Kslow}) (Göpel et al., 1999). They could also show that part of the I_{Kslow} is caused by K_{ATP} channels. A significant breakthrough could be shown by Düfer and co-workers. Studies with SK4-KO mice revealed for the first time that K_{Ca} channels of intermediate-conductance (SK4) are part of the I_{Kslow} current in pancreatic beta-cells. The I_{Kslow} current is responsible for the repolarization of the burst phase (see chapter 1.4). Pharmacological modulation of SK4 channels by TRAM-34 (1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole) led to action potential broadening and depolarized the plateau potential from which action potentials started. The broadening of action potentials leads to an elevated insulin secretion (Düfer et al., 2009).

1.3 Glucose-induced insulin secretion

Postprandial increase of blood glucose concentration leads to an increase of glucose metabolism in pancreatic beta-cells which is a prerequisite of insulin secretion. Glucose is the dominant insulin secretagogue (Henquin and Meissner, 1981). Besides glucose, other monosaccharides like fructose and mannose and also various types of amino acids and fatty acids can influence insulin secretion (Henquin and Meissner, 1981). The incretins, a group of intestinal hormones like GLP-1 (glucagon-like peptide-1) and GIP (glucose-dependent insulinotropic peptide) are additional stimuli in the presence of glucose (Ebert and Creutzfeldt, 1987).

1.3.1 Stimulus-secretion coupling (SSC)

Blood glucose concentration tightly correlates with the metabolic activity in beta-cells and the ATP/ADP ratio influence electrical activity which on the other hand controls insulin secretion (Ashcroft and Rorsman, 1989). This process is called stimulus-secretion coupling (SSC).

A postprandial increase of blood glucose concentration leads to an augmented entering of glucose into the beta-cells. In rodent beta-cells glucose enters via low-K_m (Michaelis constant) Glut-2 transporters (Johnson et al., 1990), in contrast Glut-1 and Glut-3 are the predominant glucose transporters in human beta-cells (De Vos et al., 1995; McCulloch et al., 2011). The glycolysis is the metabolic pathway that converts glucose into the intermediates pyruvate and NADH. The glucokinase (GCK) with its K_m in the range of the blood glucose concentration (~ 5 mM) is the first enzyme of the glycolysis. The GCK is the so called glucose sensor and hence the rate-determining enzyme for glucose metabolism (Lenzen and Panten, 1988). The adaptation of the necessary amount of insulin for the organism is therefore firmly regulated by the activity of the GCK. The glycolytic reduction equivalent NADH is shuttled into the mitochondria by the malate-aspartate and the glycerol phosphate shuttle systems. The inhibition of these shuttle systems completely suppresses glucose-induced insulin secretion demonstrating that only ATP resulting from the glycolytic reduction equivalents is able to influence K_{ATP} channels (Eto et al., 1999). The mitochondria are an essential link in the SSC of beta-cells. Within the mitochondria NADH is directly used by respiratory chains to pump H^+ into the intra-membrane space of the mitochondria. This process leads to an electronegative environment within the mitochondria, a prerequisite for a proper function of F_1/F_0-ATPase. Pyruvate instead, is used by the citric acid cycle and non-specialized respiratory chains to produce for the energy demands for cell function, e.g. insulin synthesis or Ca^{2+} sequestration. By contrast, the energy produced by glycolytic NADH via specific respiratory chains is linked to specific mitochondrial creatine kinases to produce a high energy cytosolic creatine phosphate (CrP) (Gerbitz et al., 1996). The CrP is shuttled via specialised electron transport chains to the K_{ATP} channels located in the membrane, where another membrane-associated creatine kinase transfers the phosphate to ADP. The produced ATP leads to the closure of K_{ATP} channels with a subsequent depolarisation of V_m (Dzeja and Terzic, 2003; Krippeit-Drews et al., 2003; Tarasov, 2006). The increase of the ATP/ADP ratio leads to a decrease of the open probability of K_{ATP} channels (Ashcroft et al., 1984; Misler et al., 1986), which leads to a depolarisation of the beta-cells via a yet unknown depolarising current (Henquin, 1978; Rorsman and Trube, 1985; Sehlin and Taljedal, 1975). When the de-

polarisation of the beta-cell exceeds the threshold for the opening of Ca_v channels, these channels open and Ca^{2+} ions enter the cell which leads to an increase of the intracellular Ca^{2+} concentration $[Ca^{2+}]_c$. The influx of Ca^{2+} ions are the direct trigger of insulin secretion via exocytosis of insulin containing vesicles. In rodent beta-cells APs are generated by L-Type Ca^{2+} channels, whereas e.g. in human beta-cells, a combined interaction between specific Na^+ and Ca^{2+} channels is observed (Braun et al., 2008).

The whole process from glucose influx until insulin secretion is called SSC. A long-lasting impairment of SSC leads to T2DM.

1.4   Membrane potential oscillations of beta-cells

A remarkable and unique feature of pancreatic beta-cells is the capability to generate membrane potential (V_m) oscillations in response to stimulatory glucose concentrations. This phenomenon leads to a pulsatile insulin secretion (Gilon et al., 1993). It is still a matter of debate whether the oscillations of insulin are important for insulin sensitivity by avoiding down-regulation of insulin receptors (Hellman et al., 2007). It is also unclear how approximately one million islets in the pancreas are able to achieve synchronized oscillatory insulin secretion. At subthreshold glucose concentrations the beta-cell V_m is silent due to its hyperpolarization to a resting potential of about ~70 mV (Ashcroft and Rorsman, 1990). An increasing glucose concentration leads to the generation of electrical activity (see chapter 1.3) arranged in AP bursts and electrical inactive interbursts (Dean and Matthews, 1970a, 1970b). The oscillatory pattern of electrical activity in beta-cells is also called slow waves. The synchronization of oscillations within an islet of Langerhans is due to a tight coupling of the cells via gap junctions (Benninger et al., 2008; Kuznetsov et al., 2005; Valdeolmillos et al., 1996). The burst and interburst length of an actively firing islet is glucose concentration dependent, i.e. with an increasing glucose concentration bursts are getting longer and interbursts shorter. The half maximal effective concentration (EC_{50}) for glucose in mouse islets is reached at ~11 mM (Henquin, 2009) whereas continuous spike activity takes place at ~20 mM. The fraction of plateau phase (FOPP) which shows the calculated percentage of burst time can be used for the quantification of the V_m oscillations.

1.4.1 Positive and negative feedback mechanisms are essential for membrane oscillations in beta-cells
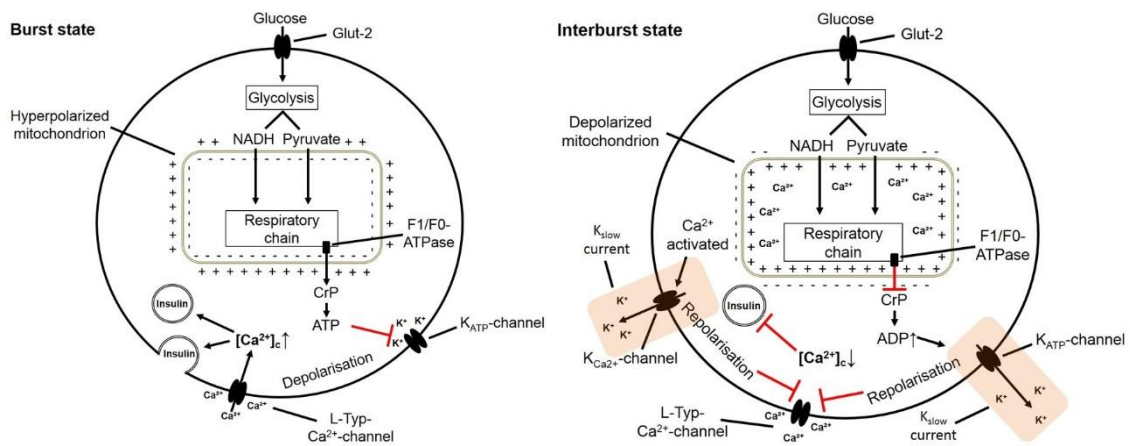
Fig 1: Burst versus interburst phase. (Left) Burst phase. Metabolism of glucose by glycolysis leads to the production of the intermediates NADH and pyruvate. Both intermediates are taken up by the mitochondria. NADH is used by respiratory chains to pump H^+ into the intra-membrane space of the mitochondria. This process leads to an electronegative environment within the mitochondria, a prerequisite for a proper function of F_1/F_0-ATPase. The metabolism of pyruvate and the reduction equivalents generated in the citric acid cycle are used to produce the bulk concentration of ATP to deliver energy for cell function. By contrast, the energy produced by NADH via specific respiratory chains is linked to specific mitochondrial creatine kinases to produce a high energy cytosolic creatine phosphate (CrP). The CrP is shuttled via specialised electron transport chains to the K_{ATP} channels located in the membrane, where another creatine kinase transfers the phosphate to ADP. The produced ATP leads to the closure of K_{ATP} channels with a subsequent depolarisation of V_m. This activates voltage sensitive L-type Ca^{2+} channels and increasing $[Ca^{2+}]_c$ triggers the exocytosis of insulin. **(Right) Interburst phase.** Electronegativity of the mitochondria enables a strong influx of the positive charged Ca^{2+} ions into the mitochondria. This initiates depolarisation of $\Delta\Psi$ which leads to a reduction of F_1/F_0-ATPase activity and consequently to a decrease of cytosolic CrP and ATP production in the close vicinity of the K_{ATP} channels. Simultaneously an additional repolarising current through K_{Ca} channels is activated. The repolarising current produced by K_{ATP} channels and K_{Ca} channels is also called I_{Kslow}. The following repolarisation inactivates voltage sensitive L-type Ca^{2+} channels. Repolarization diminishes insulin secretion and leads to the initiation of an interburst phase.

A special feature of pancreatic beta-cells is that a continuous stimulating glucose concentration generates oscillations of electrical activity, $[Ca^{2+}]_c$ and insulin se-

cretion (Drews et al., 2015). This mechanism would be impossible without a negative feedback process, which is able to transiently lower electrical activity, $[Ca^{2+}]_c$ and insulin secretion which lead to the formation of an interburst phase (Drews et al., 2015). There are reports about a positive feedback mechanism through glucose-induced increase of $[Ca^{2+}]_c$ with subsequent increase of the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$). This enhancement of ($[Ca^{2+}]_m$) then stimulates Ca^{2+}-dependent dehydrogenases and leads to a further stimulation of ATP production (Kennedy and Wollheim, 1998). This positive feedback mechanism would lead, in the presence of a stimulating glucose concentration, to a continuous production of ATP and consequently to a permanent closure of K_{ATP} channels. Other models propose a negative feedback process induced by $[Ca^{2+}]_c$ itself (Fig. 1) (Drews et al., 2015). This process can be structured into two phases, whereas Phase 1 explains the mechanisms responsible for the generation of bursts and Phase 2 the generation of interbursts (Drews 2015 et al.): (Phase 1) Stimulatory glucose concentration leads via glucose oxidation to the generation of reduction equivalents (NADH, $FADH_2$). Respiratory chains use the reduction equivalents to pump H^+ into the intra-membrane space of the mitochondria (see chapter 1.3). This process leads to a hyperpolarization of the mitochondrial membrane potential ($\Delta\Psi$). The generated H^+ gradient is necessary for the production of ATP by F_1/F_0-ATPase. The ATP which is derived from NADH is utilised by a mitochondrial creatine kinase to produce cytosolic CrP. After the transportation to the membrane via enzyme systems, another creatine kinase uses the energy rich phosphate to produce ATP. The increasing ATP/ADP ratio in the close vicinity of the K_{ATP} channels leads to a decreasing open probability of the channels, which leads to a depolarisation of V_m. This subsequently activates voltage sensitive L-type Ca^{2+} channels and this initiates Ca^{2+} influx. The increasing $[Ca^{2+}]_c$ is the direct trigger for insulin secretion. (Phase 2) Glucose concentration is still in a stimulatory range with a continued insulin secretion. During burst phase the mitochondrial $\Delta\Psi$ is hyperpolarized which leads to an electronegative environment in the interior of the mitochondria. Regarding the high $[Ca^{2+}]_c$, it comes to Ca^{2+} influx into the mitochondria with subsequent depolarisation of $\Delta\Psi$ (Krippeit-Drews et al., 2000). Depolarisation diminishes ATP production via F_1/F_0-ATPase and the open probability of K_{ATP} channels increases. At the same time an additional repolarizing current through SK4 K_{Ca} channels is activated. The K_{ATP} channel and

K_{Ca} channel currents are also called I_{Kslow} current. I_{Kslow} hyperpolarize V_m and $[Ca^{2+}]_c$ decreases. This leads to hyperpolarization of $\Delta\Psi$ and to closure of K_{Ca} channels. Thereafter ATP production increase again and Phase 1 starts again.

1.4.2 Objective

The MEA technology is an innovative technique to study the electrical activity of whole intact islets of Langerhans. We could demonstrate the `proof-of-principle´ for a MEA based method to study non-invasively the physiology and pathophysiology of islets of Langerhans (Pfeiffer et al., 2011). This study further aims to develop an assay for beta-cell damage induced by oxidative stress under controlled *ex vivo* conditions and to find appropriate protection mechanisms against it (Schönecker et al., 2014). Moreover, this study aims to develop for the first time conditions that allow electrophysiological long-term investigations of beta-cell function (Schönecker et al., 2014). The study demonstrates for the first time that MEA technology allows the analyses of electrical activity of islets isolated from human biopsies. The second part of this study focuses on the development of an MEA based recording device for semi-automatic medium-throughput recordings for islets from animal models or from human biopsies.

2   Results and discussion

An unique feature of pancreatic beta-cells is that they exhibit in whole intact islets of Langerhans oscillatory electrical activity also known as slow waves in response to glucose (Drews et al., 2010). Pfeiffer and colleagues were the first who showed oscillatory electrical activity recorded from primary, intact islets of Langerhans on top of planar microelectrodes (Pfeiffer et al., 2011). This enables for the first time calculations of the glucose dependence of FOPP using the MEA technology. For the calculation of the FOPP, it is necessary to record clearly definable oscillations with burst and interburst phases. This fluctuating electrical activity of $[Ca^{2+}]_c$ (Santos et al., 1991) and the resulting insulin secretion (Bergsten et al., 1994; Gilon et al., 1993) is an excellent marker for intact beta-cell function and metabolism (Drews et al., 2010). Previously, two other work groups reported detections of islet cell activity by extracellular electrodes, but showed only spontaneous single spike activity (Bornat et al., 2010; Palti et al., 1996).

2.1   Microelectrode array as a new approach in beta-cell physiology, pathophysiology and pharmacology

Figure 2A shows a microscopic view of a freshly isolated intact murine islet. Islets with an average diameter of ~150 μm were used for recordings on a typical glass MEA chip (Multi Channel Systems (MCS), Reutlingen, Germany) shown in Fig. 2B. For acute recordings, single islets were transferred to a MEA bath chamber, by using a holding pipette and fixed to an electrode with gentle mechanic pressure (Fig. 2C). Other exemplary recording techniques developed during my PhD thesis are shown in Fig. 2D and E (Schönecker et al., 2014). Figure 2F displays a typical MEA recording with burst activity from a single murine islet.
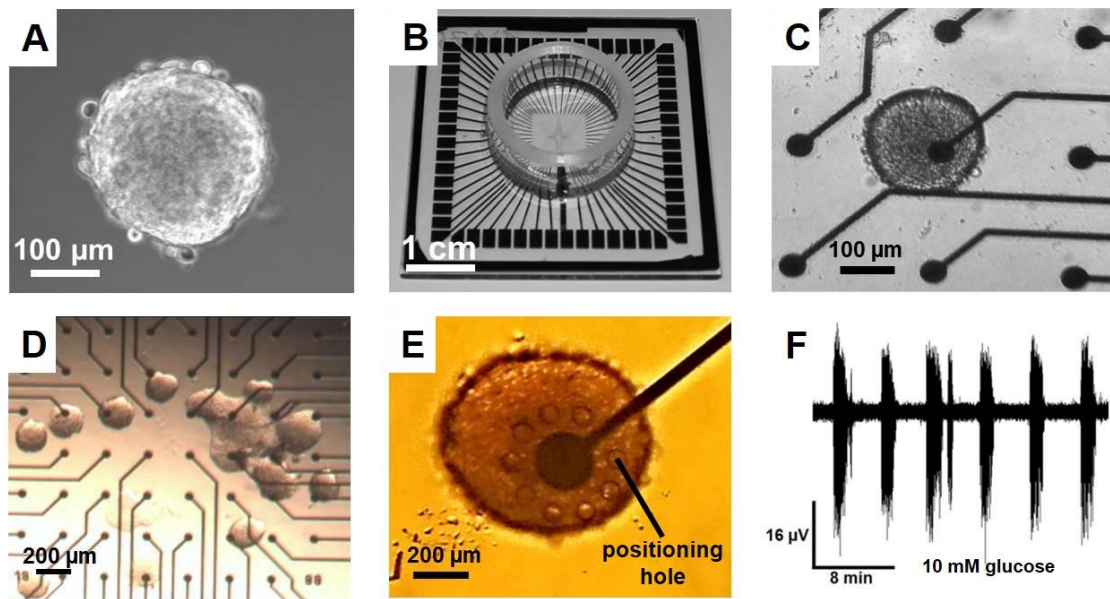
Fig.2: Islet recordings with the MEA technology. A Microscopic view of a single murine islet of Langerhans. **B** Standard microelectrode array (MEA, Multi Channel Systems). **C** Islet of Langerhans placed on top of a MEA electrode during an acute measurement. **D** Microscopic view of islets cultivated on the electrode field of a MEA for long-term recordings. **E** View of a BetaScreen electrode covered with an intact murine islet positioned via negative pressure through the positioning holes. **F** Typical field potential recording of a murine islet with characteristic oscillatory activity in 10 mM glucose.

The first important issue was to investigate whether MEA-based recordings are able to detect reliably glucose-dependent electrical activity of intact islets of Langerhans. Therefore, extracellular recordings were compared to intracellular recordings obtained by classical techniques. Figure 3A shows a trace recorded with intracellular electrodes (taken from Drews et al., 2015), whereas Fig. 3B displays an extracellular recording via the MEA technique. Importantly, the appearances of action potential voltage deflections differ from each other. Extracellular recorded signals show smaller positive and bigger negative amplitudes, whereas intracellular recorded signals show only positive voltage deflections. The reason for the different orientation of the signals is that the changes of V_m are measured by intracellular electrodes and the field potentials by extracellular electrodes. The shape of the extracellular potentials equal qualitatively the second time derivative of the intracellular V_m recordings which explains the bigger falling edge amplitudes of the signals recorded with the MEAs. There are also differences in burst length between intra- and extracellular recordings (note different time scale in Fig.3). This is may be due to differences between individual islets. Another ex-

planation could be the generation of leakage currents induced by invasive intracellular electrodes, which may influence the burst length. Quantitative analysis of the signals, i.e. the calculation of the FOPP revealed a similarity of the glucose-dependent patterns of electrical activity (see chapter 2.1.1) regardless of the recording technique (Pfeiffer et al., 2011).
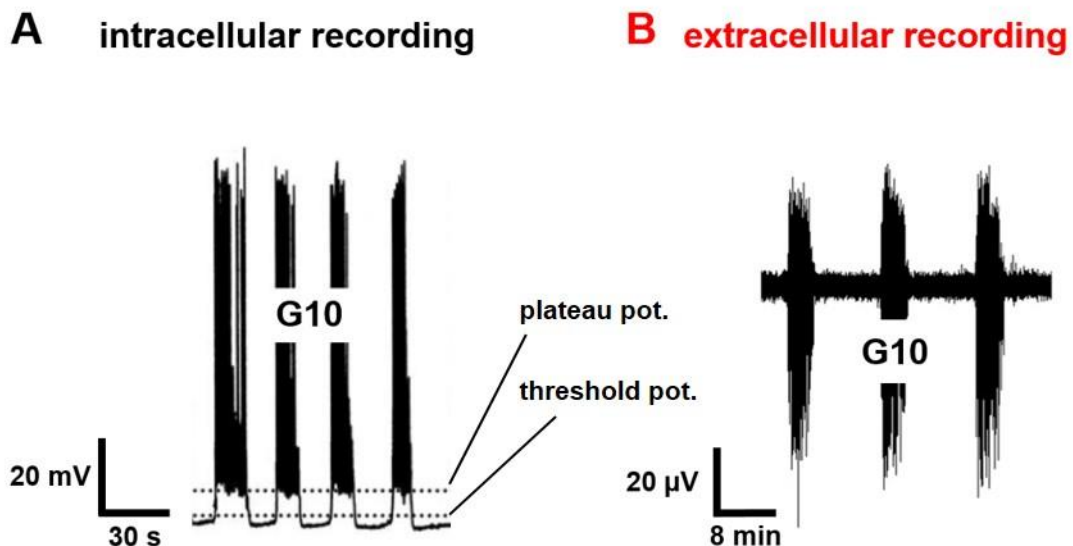
Fig.3: Comparison of intra- and extracellular recordings. **A** Exemplary data of intracellularly recorded membrane potential changes. Upward voltage deflections are Ca^{2+} action potentials defining the so-called plateau phase (figure taken from Drews et al., 2015). **B** Field potential recording on a MEA electrode. The recording is qualitatively comparable to intracellular measurements. A and B in 10 mM glucose.

2.1.1 Glucose-induced electrical activity of beta-cells

Increasing extracellular glucose concentration leads, according to the consensus model of the stimulus-secretion coupling (Ashcroft and Rorsman, 1989), to Ca^{2+} APs with voltage deflection bursts which are detectable with MEA electrodes (Pfeiffer et al., 2011). The characteristic pattern of electrical activity can be divided in two phases. The first phase is characterised by a very long burst followed by an extended silent period. Thereafter during the second phase, the typical oscillations of V_m take place (Drews et al., 2015). For the analyses of the fluctuating electrical activity it is necessary to quantify the signals. The calculation of the FOPP is shown in Fig. 4C, whereas Figure 4A shows two representative MEA recordings from islets in 10 (lower trace) and 15 mM glucose (upper trace). Note the burst rate and hence the FOPP is higher in 15 mM compared to 10 mM glucose. Glucose concentration response curve plotted as FOPP [%] is shown in

Fig. 4B, measured with MEA electrodes. The provided values range from 0% without activity for 3 mM glucose (n=10) to 100% which represents continuous spiking in 30 mM glucose (n=5). FOPP values increased with 6, 8, 10 and 15 mM glucose to 22 ± 3% (n=5), 36 ± 4% (n=5), 38 ± 3% (n=8), and 56 ± 4% (n=11), respectively. The half-maximal activity (50% FOPP) revealed 12 ± 1 mM, which is in agreement with data recorded with intracellular microelectrodes (11 mM) (Henquin, 2009) and clearly shows that extracellular MEA recordings from whole intact islets exhibit the same glucose dependence as recorded with intracellular electrodes. Interestingly, there is no electrical activity observable from alpha-cells. This is due to the fact that alpha-cells display within intact islets the opposite glucose dependence as beta-cells, i.e. inhibition at high and activation at low glucose (Rorsman et al., 1989). During the application of 3 mM glucose the electrical baseline appears (electrical noise) which is a random fluctuation in all electronic circuits. If alpha-cell activity would contribute to the baseline, it should be silenced shortly before the beta-cell activity appears after increased glucose concentration, but no changes were detected. In contrast to this Raoux and colleagues recorded electrical activity from dissociated alpha- and beta-cells revealed from mouse islets on the same MEAs but on different electrodes (Raoux et al., 2012). This can only be achieved when islets, prior to the cultivation, are dissociated to cell cluster or single cells via trypsin. Pfeiffer and Schönecker and colleagues showed that MEA recordings with whole intact islets display only beta-cell activity (Pfeiffer et al., 2011; Schönecker et al., 2014, 2015). In addition, the appearance of oscillations occur rarely in clustered beta-cells and never in dissociated single beta-cells which underlines the importance to record from whole intact islets.
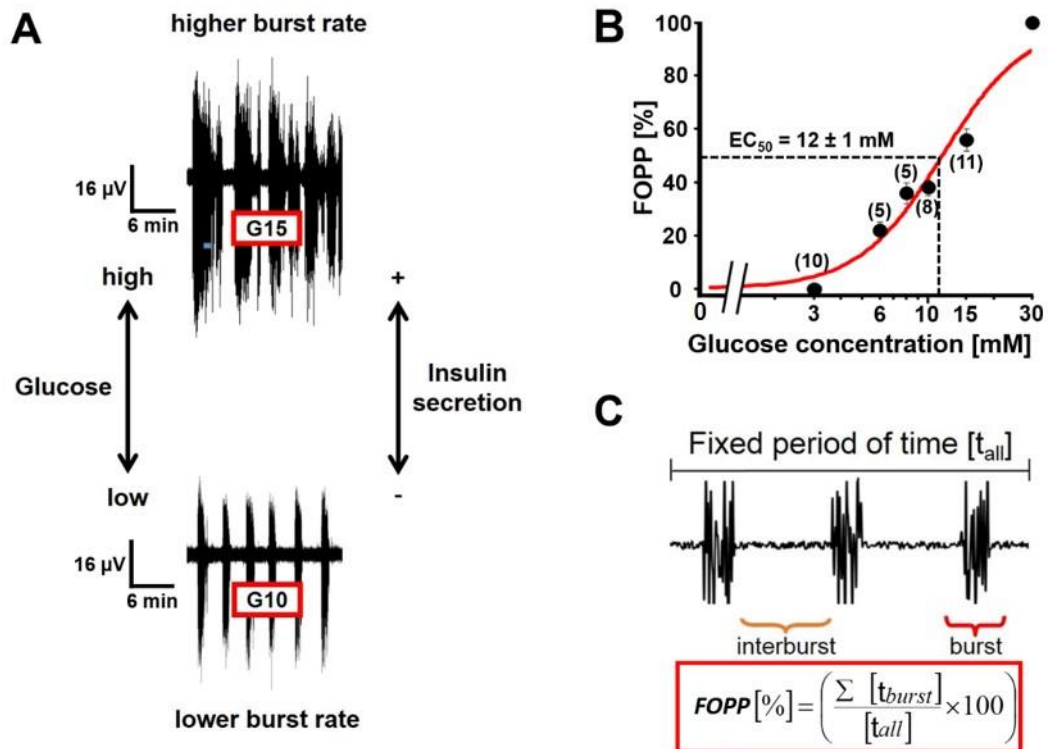
Fig.4: Glucose-induced electrical activity and calculation of the fraction of plateau phase (FOPP). A Two MEA recordings from islets in 10 and 15 mM glucose. Electrical activity is higher in 15 than in 10 mM glucose. **B** Glucose concentration response curve plotted as FOPP [%] against glucose concentration [mM] (taken from Pfeiffer et al., 2011). In 3 mM glucose no activity was detected while 30 mM led to continuous activity. **C** Graphic representation of the fraction of plateau phase (FOPP) quantifying beta-cell activity.

2.1.2 Effects of ion channel modulators and simultaneous recordings of electrical activity and [Ca^{2+}]$_c$

To further validate and proof the reproducibility of the signals, several ion channel modulators were tested on murine islets. The modulation of K$_{ATP}$ channels with diazoxide (100 µM) a potent potassium channel activator resulted in a silencing of the oscillatory pattern (Fig. 5A). The activation of K$_{ATP}$ channels via diazoxide leads to an increase of the hyperpolarising K$^+$ outward current and hence to the closure of voltage sensitive L-type Ca^{2+} channels (Dunne et al., 1987; Henquin and Meissner, 1982; Trube et al., 1986). Thus diazoxide is often used to counter hypoglycaemia in diseases such as insulinoma or CHI (Gill et al., 1997). The oscillatory pattern can reappear after the addition of the K$_{ATP}$ channel blocker tolbutamide (150 µM) a sulfonylurea derivative (Fig. 5A). Sulfonylureas were an important class of antidiabetic drugs used to treat T2DM and act by increasing insulin secretion via the closure of K$_{ATP}$ channels (Gillis et al., 1989). Figure 5B

shows the blockage of oscillations by nifedipine which is a dihydropyridine L-type Ca^{2+} channel blocker (Vater et al., 1972). The blockage with nifedipine clearly displays the predominant role of L-type Ca^{2+} channels for the generation of APs in murine islets. Figure 5C compares simultaneous electrical recordings on a MEA with $[Ca^{2+}]_c$ recordings via Ca^{2+} imaging. For this experiment the MEA was placed on an inverted microscope. The islet was incubated with the Ca^{2+} chelator fura-2 and then placed with mechanic pressure on top of a MEA electrode. Subsequent excitation with 360 and 380 nm wavelength through the glass MEA revealed changes of $[Ca^{2+}]_c$ and at the same time glucose-induced electrical activity was measured by MEA electrodes. Importantly, each burst of APs was accompanied by a peak of $[Ca^{2+}]_c$ (Fig. 5C). This findings were already shown by other groups who could show that $[Ca^{2+}]_c$ (Santos et al., 1991) and insulin secretion (Bergsten et al., 1994; Gilon et al., 1993) are tightly coupled to the fraction of time beta-cells are bursting, underlining that the FOPP is an excellent marker for insulin release.
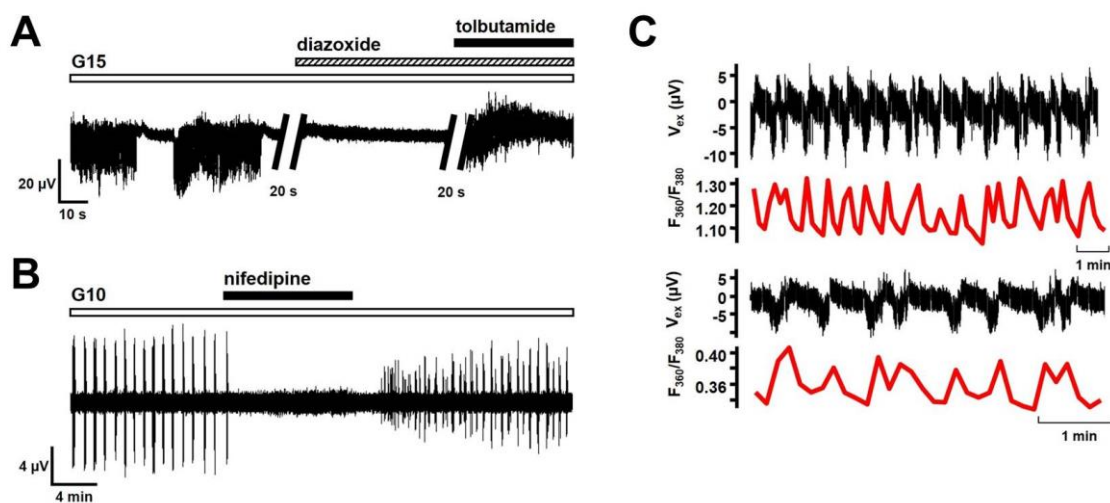
Fig.5: Ion channel modulation and simultaneous recordings of electrical activity and $[Ca^{2+}]_c$. A Recording of electrical activity in 15 mM glucose. Application of the K_{ATP} channel opener diazoxide (100 μM) results in a silencing of the oscillatory pattern. Additional application of the K_{ATP} channel blocker tolbutamide (150 μM), on the other hand, restored the electrical activity (FOPP in % in G15 = 61, G15+diazoxide = 0 and G15+diazoxide+tolbutamide = 100. One representative of 11 MEA recordings. **B** Application of the L-type Ca^{2+} channel blocker nifedipine (n=4) silenced oscillations recorded in 10 mM glucose (FOPP in % in G10 = 42, G10+nifedipine = 0 and after washout = 45). **C** Two examples of simultaneous recordings of electrical activity and $[Ca^{2+}]_c$. The upper traces (black) show glucose-induced electrical activity, and the lower traces (red) are recordings of $[Ca^{2+}]_c$. Importantly, each burst of electrical activity is accompanied by a peak of

$[Ca^{2+}]_c$. Glucose concentration was 10 mM (FOPP in % upper black trace = 40; FOPP in % lower black trace 38). (Fig.3 C in cooperation with Prof. Dr. Martina Düfer).

2.2   A superoxide dismutase (SOD) mimetic protects beta-cells against acute oxidative stress

An important milestone of my thesis was the development of an *in vitro* environment capable to induce oxidative stress on MEAs. It is well accepted that oxidative stress, as a result of excessive fuel intake (modern lifestyle) within pancreatic beta-cells is a key event in the development and progression of T2DM (Poitout and Robertson, 2008). That oxidative stress impairs beta-cell function and inhibits glucose-stimulated insulin secretion is already known (Akesson and Lundquist, 1999; Krippeit-Drews et al., 1999). Since, the equipment with antioxidant enzymes (e.g. catalase (Cat), glutathione peroxidase (Gpx) and superoxide dismutase (SOD)) seems to be low in beta-cells (Lenzen et al., 1996), it would be of great importance to find strategies to prevent oxidative stress. The development of an *in vitro* model capable to induce a defined stress level on top of MEA electrodes would help to understand the effect of stress-induced damage to beta-cells and to find strategies against it. An important question was whether the islet recordings with the MEA technology are sensitive enough to allow the detection of small changes in electrical activity induced by stress. It would be even more important to be able to detect protective effects against stress induction with the help of MEA recordings. In the present study I investigated hydrogen peroxide (H_2O_2) induced changes in electrical activity of mouse beta-cells recorded on MEAs. H_2O_2 is an oxygen species which generates oxidative stress within pancreatic beta-cells (Poitout and Robertson, 2008). The effect of different H_2O_2 concentrations on the electrical activity of islets was examined. Figure 6A shows one typical recording of the application of 300 µM H_2O_2 in the presence of 10 mM glucose. The decreasing effect of H_2O_2 on electrical activity is clearly detectable with the MEA technology. The concentration-response curve shows the decreasing effect of H_2O_2 on the FOPP (Fig. 6B), (n=3-14. **P ≤ 0.01 *** P ≤ 0.001 compared to control without H_2O_2). The results illustrate clearly that H_2O_2 markedly contribute to changes in electrical activity. These findings are in line with another study who showed that H_2O_2 is one of the reactive oxygen species which strongly

contributes to an increasing K_{ATP} current (Krippeit-Drews et al., 1994). Further-more, alterations in electrical activity or K_{ATP} channel current in the presence of H_2O_2, nitric oxide (NO) or other reactive oxygen species (ROS) and reactive ni-trogen species (RNS) donors have been described (Drews et al., 2000; Krippeit-Drews et al., 1994, 1995, 1999; Nakazaki et al., 1995; Tsuura et al., 1994). The next step was to investigate whether the membrane-permeable SOD mimetic, tempol (Fleenor et al., 2012), is able to protect beta-cells against H_2O_2-induced effects on electrical activity. To avoid a direct extracellular contact of tempol and H_2O_2 the islets were pre-incubated with tempol (1 mM) prior to the application of 300 µM H_2O_2. As shown in Fig. 6C and D a 30 min pre-incubation of 1 mM tempol (n=14) protected the islets against the application of 300 µM H_2O_2. In addition tempol has also a protective effect on insulin secretion shown in Fig. 6E (n=6). There are differences in the effectivity of the H_2O_2 effect on insulin secretion and electrical activity which is most likely due to slight differences in the experimental procedure (perfusion for the measurement of electrical activity vs. 1 hour steady-state incubation for insulin secretion). The results clearly show that tempol has a protective effect on insulin secretion by avoiding the damaging effects of H_2O_2 on SSC. This is in agreement with previous observations that up-regulation of anti-oxidant enzymes protects beta-cells against oxidative stress (Gier et al., 2009). Moreover, the data demonstrate that SOD could be an important key player in the antioxidant defence of beta-cells which seems to be even more important than Gpx and Cat. This can be explained by the fact that the application of SOD alone is already effective and protects against oxidative stress. Interestingly, the main substrate of SOD is not H_2O_2, but rather the superoxide anion radical (O_2^-) which can react with critical cellular targets e.g. with NO to toxic peroxynitrite. The question that arise is, how does SOD protect against the application of H_2O_2. It is presumed that the tempol-dependent protection is due to the degradation of H_2O_2–induced ROS production including O_2^-. It is known that H_2O_2 leads to a partial depolarization of the mitochondria (Nakazaki et al., 1995) and a decrease of $\Delta\Psi$ is assumed to increase ROS production (Krippeit-Drews et al., 1999). Moreover, ROS in general, including H_2O_2 is known to induce mitochondrial ROS release (Zorov et al., 2006). This mechanism is well known in cardiomyocytes and involves the mitochondrial permeability transition pore/or the anion channel of the inner mitochondrial membrane. In both cases the generation of O_2^- in the

mitochondria would increase and intensify ROS generation and action. We hypothesize that this vicious circle is stopped by the SOD mimetic (Schönecker et al., 2014). The results show the successful establishment of an oxidative stress model on top of MEAs and demonstrate the protective effect of the SOD mimetic tempol on electrical activity of beta-cells and insulin secretion.
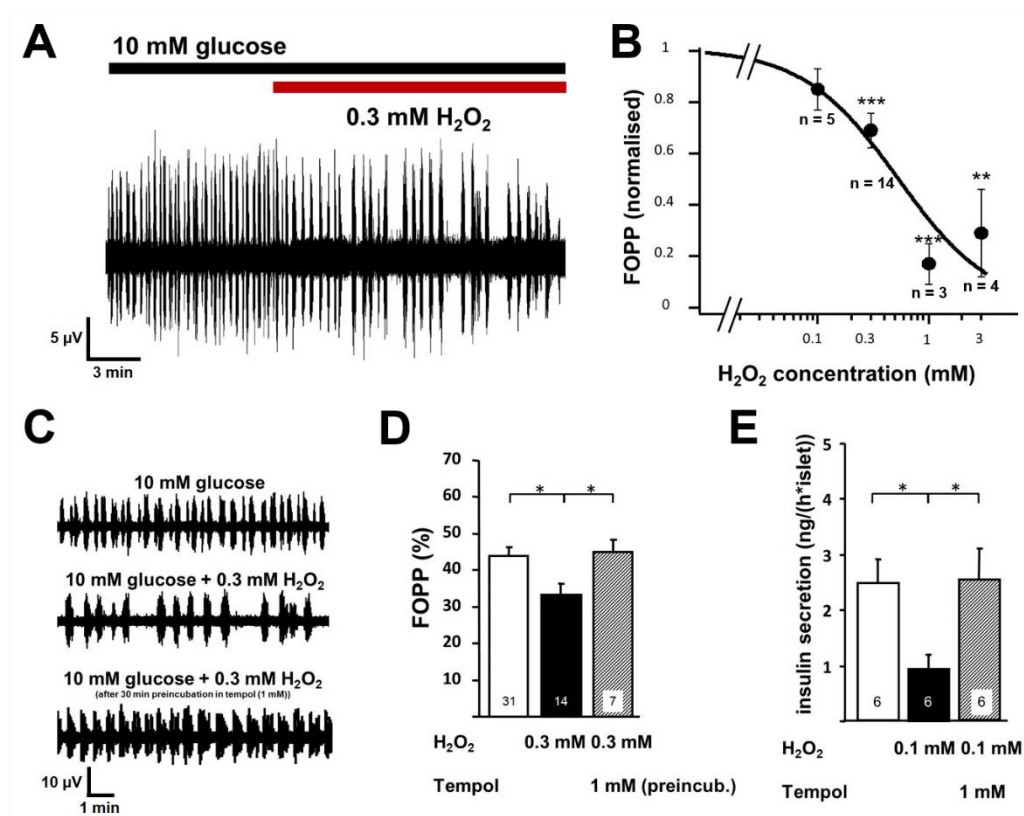
Fig.6: Effect of H_2O_2 on electrical activity in mouse islets and the protective effect of tempol against H_2O_2-induced reduction of electrical activity. A Typical extracellular MEA recording of glucose-induced oscillations before and during application of 0.3 mM H_2O_2 (n=14). **B** Dose-response curve showing the decrease of the FOPP in the presence of H_2O_2 (n=3-14. **$P \leq 0.01$ *** $P \leq 0.001$ compared to control without H_2O_2). **C** Original recordings in 10 mM glucose (upper trace), 10 mM glucose and 0.3 mM H_2O_2 (middle trace) as well as in 10 mM glucose and 0.3 mM H_2O_2 after preincubation in 1 mM tempol (lower trace). The protective effects of tempol on FOPP **(D)** and insulin secretion **(E)** (Fig.6 E performed from the workgroup of Prof. Dr. Gisela Drews, University of Tübingen).

2.3   Establishment of a long-term culture of intact murine islets on MEAs

The development and progression of the pathogenesis of T2DM is a long-lasting process. And as mentioned above, all current electrophysiological techniques used in beta-cell research are invasive which makes *in vitro* long-term investigations of islets impossible. Therefore, it would be of great advantage for islet research to overcome these limitations and to find a way to develop culture conditions for long-term investigations of intact islets in order to come as close as possible to the *in vivo* situation. Therefore, the next aim was to develop a system allowing a long-term culture of intact islets on MEAs. The first challenge was to find an appropriate substrate for the islets so that they can attach on the MEA surface. Matrigel (Corning Life Science) is a gelatinous protein mixture produced by Engelbreth-Holm-Swarm mouse sarcoma cells and is similar to the extracellular matrix found in most tissues (Hughes et al., 2010). Matrigel allows the coating of substrates, such as the MEA surface, having a thin layer with a thickness in the range of a few micrometer (Hughes et al., 2010). This thin Matrigel layer turned out to be a very good substrate for the cultivation of islets on top of MEAs. Figure 7 (upper left) shows a microscopic view of an electrode field of a MEA with cultivated islets grown on top. This example shows islets already cultured for 12 days on the MEA surface. Importantly, they still display glucose-dependent electrical activity comparable to acute recordings. Typical oscillations simultaneously recorded from seven islets (red circles) are presented aside (black traces), showing the increase in electrical activity with increasing glucose concentrations (Fig. 7).
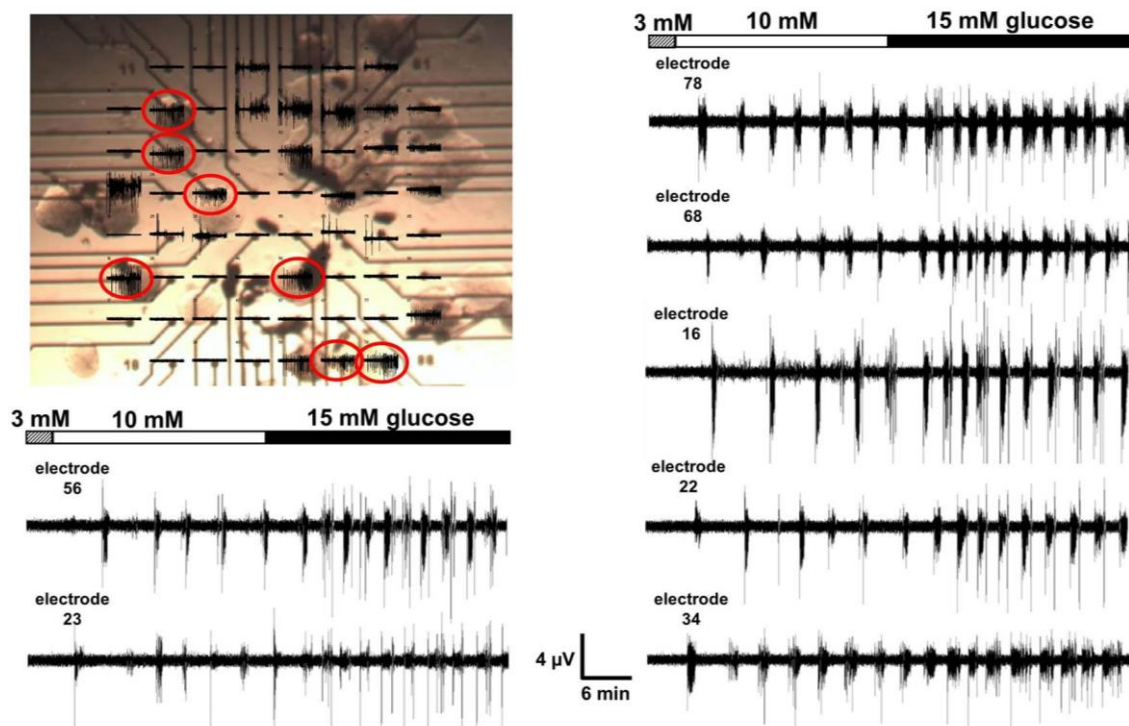
Fig.7: Parallel recordings of beta-cell activity from different islets after long-term cultivation (12 days *in vitro*) on MEAs. Upper left: Microscopic view of islets cultivated on the electrode field of a MEA. Typical oscillations from seven islets (red circles) are presented aside, showing the increase in electrical activity with increasing glucose concentrations. Note: the activity was recorded from islets already cultured for 12 days on top of the MEA surface. The FOPP in 10 mM glucose amounted to 22 ± 3% (n=7) and for 15 mM glucose 40 ± 4% (n=7).

Next it was highly important to elucidate whether the electrical activity of islets is preserved in culture. Therefore, I examined the glucose-induced electrical responsiveness of the islets over several weeks and compared the data with the results of the previously obtained acute recordings. Two exemplary traces from islets cultured 6 and 34 days on a MEA are shown in Fig.8A. During the application of 3 mM glucose no activity was detectable, whereas an increase to 10 and 15 mM glucose induced electrical activity even after 34 days in culture. In a series of measurements electrical activity of cultured islets was recorded in the presence of 10 and 15 mM glucose for different days of culture (Fig. 8B). Differences of electrical activity recorded in 10 mM compared to 15 mM glucose was clearly distinguishable, showing the preservation of electrical activity up to 34 days in culture. Quantification of the FOPP in the presence of 10 and 15 mM glucose for different days of culture. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Statistical differences between the FOPP at 10 and 15 mM glucose at each culture day. The n-values

within the columns indicate the number of islets. 5 different preparations are included in this series of experiments. To examine whether there are culture time-dependent alterations in electrical activity, the FOPP was pooled for experiments recorded in the first week (day 6 and 7) and compared to recordings obtained in the last week (day 33 and 34). The FOPP calculated for the first week amounted to $32 \pm 3\%$ (n=8) for 10 mM glucose and $70 \pm 6\%$ (n=8) for 15 mM glucose. The calculations for the last week revealed $36 \pm 2\%$ (n=7) for 10 mM glucose and $72 \pm 5\%$ (n=7) at 15 mM glucose. This data clearly show that the FOPP did not change over time in culture and is even after 34 days comparable to acute recordings carried out with freshly isolated islets (Fig. 8B right). The establishment of a long-term culture of intact murine islets on MEAs is the first electrophysiological approach allowing long-term investigations of whole intact islets (Schönecker et al., 2014). Raoux and co-workers cultivated clonal INS-1E cells on MEAs and reported a reduced AP frequency after 3 days at elevated glucose concentration (Raoux et al., 2012). It can be speculated, that the reduction of the spike rate of INS-1E cells over the cultivation time is due to differences in the cellular environment in comparison to intact islets. To investigate changes in islet cells in a T2DM promoting environment it is a great advantage to record from structurally intact primary islets to come as close as possible to the *in vivo* situation. Furthermore, it is possible to study the oscillatory activity of the same islet on different days, which enables to monitor e.g. long-term effects of anti-diabetic drugs. Another beneficial effect is the increase of throughput. The rate of successful experiments could be enhanced by a factor of approximately 8 compared to a single islet recording with the acute method. Theoretically 60 islets could be placed and recorded on a standard MEA simultaneously. To obtain this amount of recordings per MEA, further advancements are necessary. The most important step would be to develop a surface patterning which allows a targeted placement of the islets on individual electrodes. First prototypes are already developed and will be tested at the NMI (The Natural and Medical Sciences Institute at the University of Tübingen).

This new development will revolutionise electrophysiological *in vitro* experiments and paves the way to perform long-term recordings of electrical activity with low concentrations of redox-active compounds or diabetes-inducing agents, and also

to study progressive changes evoked by gluco-, lipo- or glucolipotoxicity. Moreover, the method allows developing co-culture experiments with other cell types or tissues to investigate whether there are influences on electrical activity.
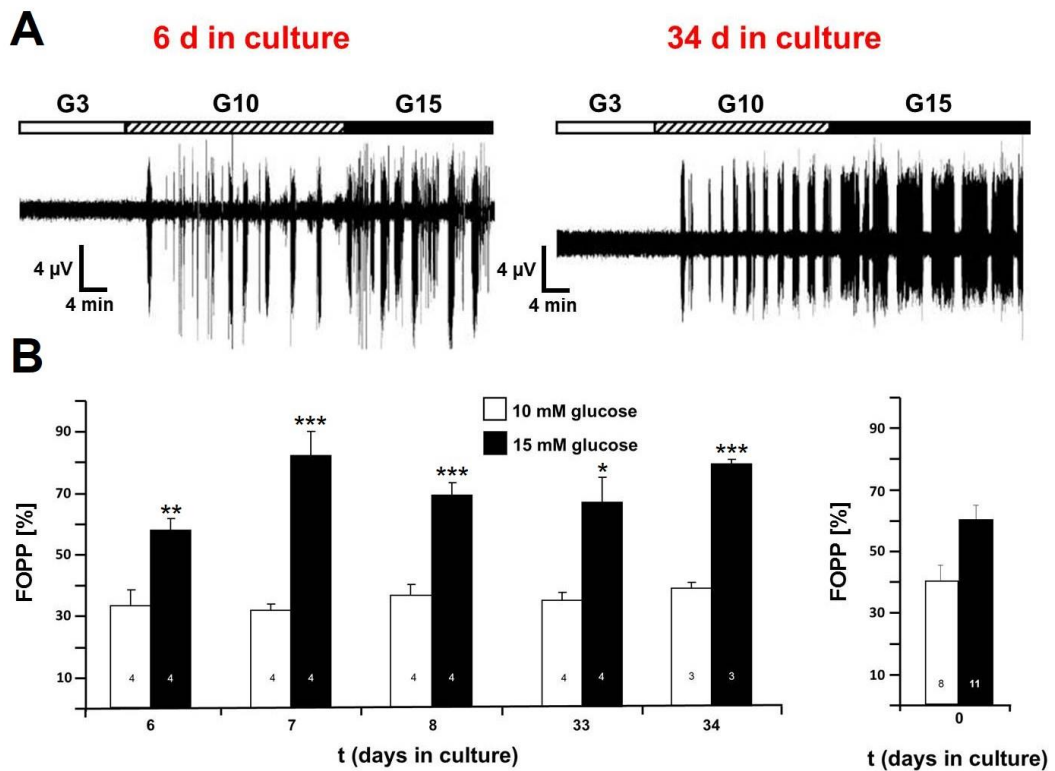
Fig.8: Electrical activity of islets is preserved in culture. A Two representative recordings from islets cultured already 6 and 34 for days on a MEA. Electrical activity was induced by the application of 10 and 15 mM glucose, whereas no activity was detectable with the sub-threshold concentration of 3 mM glucose. **B (left)** FOPPs for glucose concentrations of 10 and 15 mM are not significantly altered up to 34 days in culture and are comparable to **(right)** FOPPs recorded from freshly isolated islets. *$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Statistical differences between the FOPP at 10 and 15 mM glucose at each culture day. The n-values within the bars indicate the number of islets. 5 different preparations are included in this series of experiments.

## 2.4   Human islets exhibit electrical activity on MEAs and on the new developed BetaScreen device

Most of the fundamental knowledge about pancreatic beta-cells was achieved from research with animal models. This is due to limited access to human biopsies, and if available, the quality and the number of the islets are often very low. Nevertheless, in recent years successively more and more studies have been

made with human beta-cells. These studies demonstrated various important differences between rodent and human beta-cells. This underlines the importance to further improve the current scientific knowledge about human islets. An important tool to improve these investigations could be the MEA technology. This study demonstrates for the first time that the MEA technique is suitable to record electrical activity from human islets (Schönecker et al., 2015).

Human islets were obtained either from biopsies taken during pancreatic surgery or from organ donors isolated according to the Edmonton Protocol. To verify whether the MEA technology is able to record electrical activity from human islets, different recording methods were tested. Figure 9 shows microscopic views of different recording techniques tested with human islets. The left photo (Fig. 9A) displays the mechanical placement of an islet via a holding pipette during an acute recording. A human organotypic pancreatic slice on top of a MEA is shown in Fig. 9B fixed by a platinum grid and a human islet culture on the electrode field of a MEA can be seen in Fig. 9C. An exemplary recording of electrical activity of a human islet received from an adult donor with the acute technique is shown in Fig. 9D. During the application of 1 mM glucose no activity could be recorded whereas electrical activity started after the application of 10 mM glucose. The additional application of the sulfonylurea tolbutamide (100 µM) increased the glucose-induced activity clearly. In other recordings, spike activity could be inhibited by diazoxide (Schönecker et al., 2015). Interestingly, electrical activity could also be obtained from a pancreatic slice of a child with CHI. This demonstrates that MEA recordings with human pancreatic tissue is not depending on the age of the donor. Importantly, the recordings were obtained from different isolation protocols. Either by an isolation protocol similar to the one which I used for the isolation of primary mouse islets, or from organ donors isolated according to the Edmonton Protocol (Shapiro et al., 2000). The Edmonton Protocol for islet cell transplantation was developed at the University of Alberta Hospital (Edmonton, Canada) to guarantee a quality standard for the isolation of human islets prior to the transplantation into patients with type 1 diabetes. The recording which can be seen in Fig. 9D was obtained from an islet isolated according to the Edmonton Protocol. The success rate of the human islet recordings with the MEA-technology was higher with the Edmonton Protocol. We hypothesize that the isolation process

and therefore the quality of the human islets is a very critical point for successful recordings.
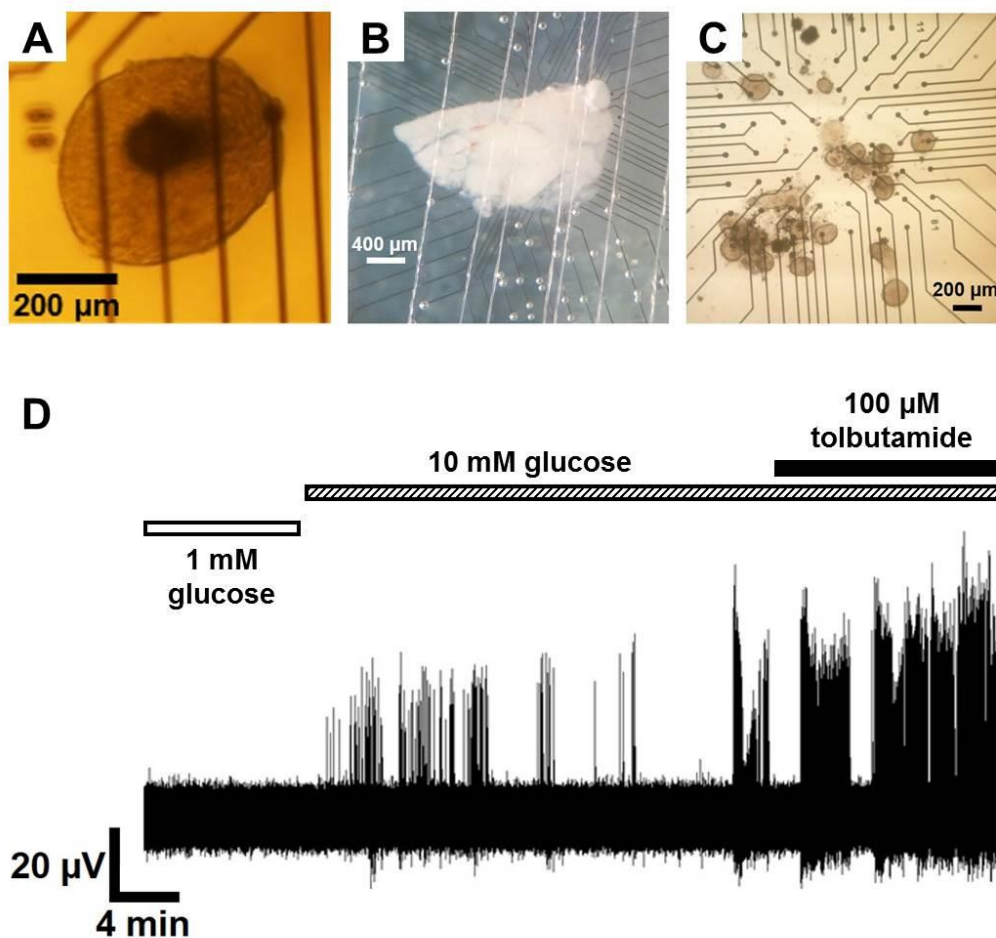
Fig. 9: Human islet activity can be recorded with the MEA technology. A Microscopic view of an islet placed by mechanical pressure with a holding pipette during an acute measurement. **B** Pancreas slice from a child with CHI on top of a MEA. **C** Cultivated human islets on MEA electrodes. **D** Electrical activity of a human islet of Langerhans obtained from an adult donor. The application of 10 mM glucose (FOPP in % = 35) evoked spike activity and additional application of tolbutamide further increased the spikes (FOPP in % = 85).

As recent studies demonstrated that there are important differences between rodent and human beta-cells (Rorsman and Braun, 2013). One important example is the effect of the voltage-dependent Na^+ channel blocker tetrodotoxin. Figure 10A shows a strong inhibition of TTX (300 nM) on the electrical activity of a human islet (n=11) (Schönecker et al., 2015). Interestingly, the effect of TTX did not completely diminish spike activity and the effect was reversible. Figure 10B shows a comparable recording on a murine islet. In contrast to human islets, TTX

did not influence electrical activity of mouse islets (n=3) (Schönecker et al., 2015). These findings are in line with the data reported by Braun et al. (Braun et al., 2008), were he could show that the gating of voltage-dependent Na^+ channels plays an important role for electrical activity in human islets. The application of 300 nM TTX which is a selective and effective inhibitor of voltage-gated Na^+ channels, reduced burst activity largely and reversibly as shown in Fig. 10A. Moreover, the results show clearly that TTX inhibits burst activity not completely and that there is activity remaining. Responsible for the remaining activity are most likely still active L-type Ca^{2+} channels. L-type Ca^{2+} channels and Na^+ channels are important for the initiating of the APs. This can be confirmed through the inhibitory effects of the Ca^{2+} channel blocker isradipine together with TTX on insulin secretion in human islets (Rorsman and Braun, 2013). The comparison to mouse islets (Fig. 10A and B) shows one of the important differences between mouse and men within the SSC of beta-cells. TTX exhibits a strong effect in human islets, whereas mouse islets remain unaffected. These findings are in line with Plant (Plant, 1988) who showed that Na^+ channels of mouse beta-cells are not functional at physiological membrane potentials.
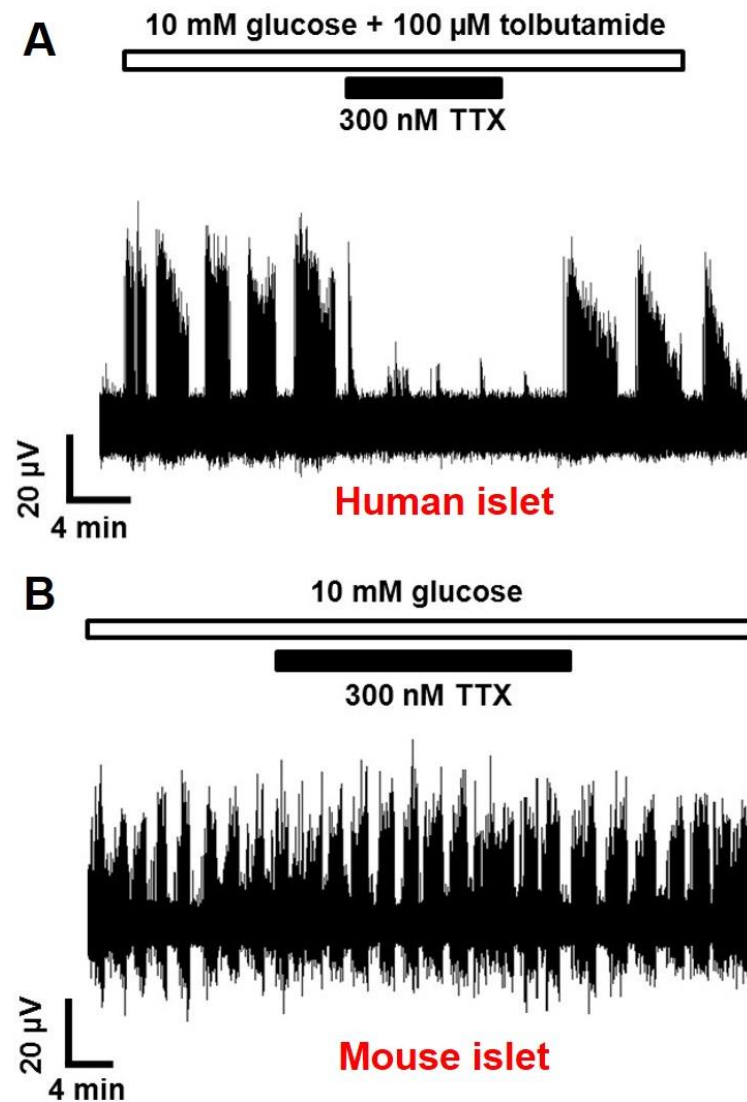
Fig.10: Comparison of the tetrodotoxin (TTX) effect on electrical activity of human vs. mouse islets. A The application of 300 nM TTX almost completely diminished electrical activity of a human islet (n=11) in the presence of 10 mM glucose and 100 µM tolbutamide (Spike activity in 10 mM glucose 1047 ± 314, glucose and TTX 199 ± 90 and after the washout of TTX 779 ± 301). **B** TTX is without effect on electrical activity of a murine islet in the presence of 10 mM glucose (n=3) (FOPP in % for 10 mM glucose = 61 ± 9, FOPP in % in 10 mM glucose and 300 nM TTX = 60 ± 8 and after the washout of TTX = 63 ± 9).

2.4.1 Transfer of human islets of Langerhans to the BetaScreen approach

The BetaScreen (see chapter 3) is a very useful new electrophysiological medium throughput screening device for whole intact murine islets of Langerhans. As well as for mouse islets, an increase of throughput for the human islet recordings would be of great scientific interest. This study obtained for the first time simultaneous electrophysiological recordings of human islets by means of the BetaScreen device. The recordings with the BetaScreen device enabled to record

a high number of primary human islets in a short time. This was highly important since the quality of human islets decreases over the time. The declining quality of the human islets makes it very difficult to obtain an adequate amount of measurements during the short timeframe which exists for the recordings. The increase of successful recordings paved the way to quantify pharmacological modulations of the electrophysiological recorded signals and revealed for the first time a glucose concentration-response curve obtained from human islets on a MEA system.

For the quantification of the electrical activity from human islets recorded with the BetaScreen device, the number of single spike activity (spikes/5min) was used, due to the absence of oscillations. The validation studies with tolbutamide and diazoxide showed the existence of functional K_{ATP} channels. Fig. 11A shows the effects of diazoxide (100 µM, n=10) and tolbutamide (150 µM, n=10) on spike activity. Diazoxide reduced the mean spike activity from 833 ± 339 spikes/5min (10 mM glucose) to 8 ± 4 spikes/5min. The additional application of tolbutamide restored spike activity up to 876 ± 310 spikes/5min. The quantification of the TTX effect is shown in Fig. 11B. Spike activity was reduced from 1047 ± 314 to 199 ± 90 spikes/5min after the application of 300 nM TTX (n=11). The washout led to an increase of spike activity to 779 ± 301 spikes/min (n=11). The glucose concentration-response curve of the spike activity is shown in Fig. 11C. Spike activity increased from 118 ± 35 (n=11), 142 ± 31 (n=11), 368 ± 58 (n=31), 710 ± 90 (n=11) to 945 ± 156 spikes/5min with 1, 3, 5, 8 and 10 mM glucose, respectively. The Hill equation fitting revealed an EC_{50} value of 8.66 ± 3.02 mM glucose which is lower than in mouse islets. The glucose responsiveness in human islets recorded with the MEA-technology is in line with other reports showing that the EC_{50} value of human beta-cells (~ 6 mM) is smaller than in mouse beta-cells (~ 11 mM) (Rorsman and Braun, 2013). This differences explain the fact that the nonfasting plasma glucose concentration is lower in human (~5 mM) (Frayn 2010) than in mice (7-10 mM) (Li et al., 2009; Remedi et al., 2011). That the higher glucose sensitivity of human islets is due to intrinsic beta-cell properties, could be shown by Ricordi and co-workers who transplanted human islets into diabetic mice. The result was that the insulin secretion of the human beta-cells regulated plasma glucose at the normal nonfasting glucose concentration for humans rather than at the nonfasting glucose concentration for mice (Ricordi et al., 1991). Example

of an insulin secretion experiment is shown in Fig.11D. Glucose-induced insulin secretion was obtained from adult human islets. The islets secreted only a small percentage of the stored insulin which was less than 0.06% of 10 ± 1.6 μU/10 islets (n=7) and glucose-induced insulin secretion did not become significant showing a low responsiveness of this preparation to glucose.

As already mentioned above, the recordings with human islets revealed only seldom electrical oscillations in comparison to mouse recordings. Therefore, I used the quantification of single spikes per five minutes for the analyses. A similar observation is shown by Rorsman and Braun (Rorsman and Braun, 2013), where 58% of the recordings showed single spike activity and only 26% oscillatory activity. The question arise of whether such a behaviour reflects the physiological behaviour of the human islets, or if this is only seen in *in vitro* experiments due to possible stress factors after the isolation process of the islets. It is a fact that more *in vitro* recordings from human islets are necessary to answer this kind of questions and the MEA technology with the capability of higher throughput recordings can help to generate answers.

In conclusion, the MEA method emerges as a new important tool for the investigation of rodent and human islets *in vitro*. The MEA technique will increase the success rate of the recordings which is important due to limited access of, e.g. human pancreatic tissue probes. The development of murine islet cultures on top of MEA electrodes for long-term recordings can also be transferred to human islets. And last but not least, human islet recordings with the medium-throughput BetaScreen device (see chapter 3) can be used for pharmaceutical drug screenings and even to test human islets for metabolic integrity before transplantation.
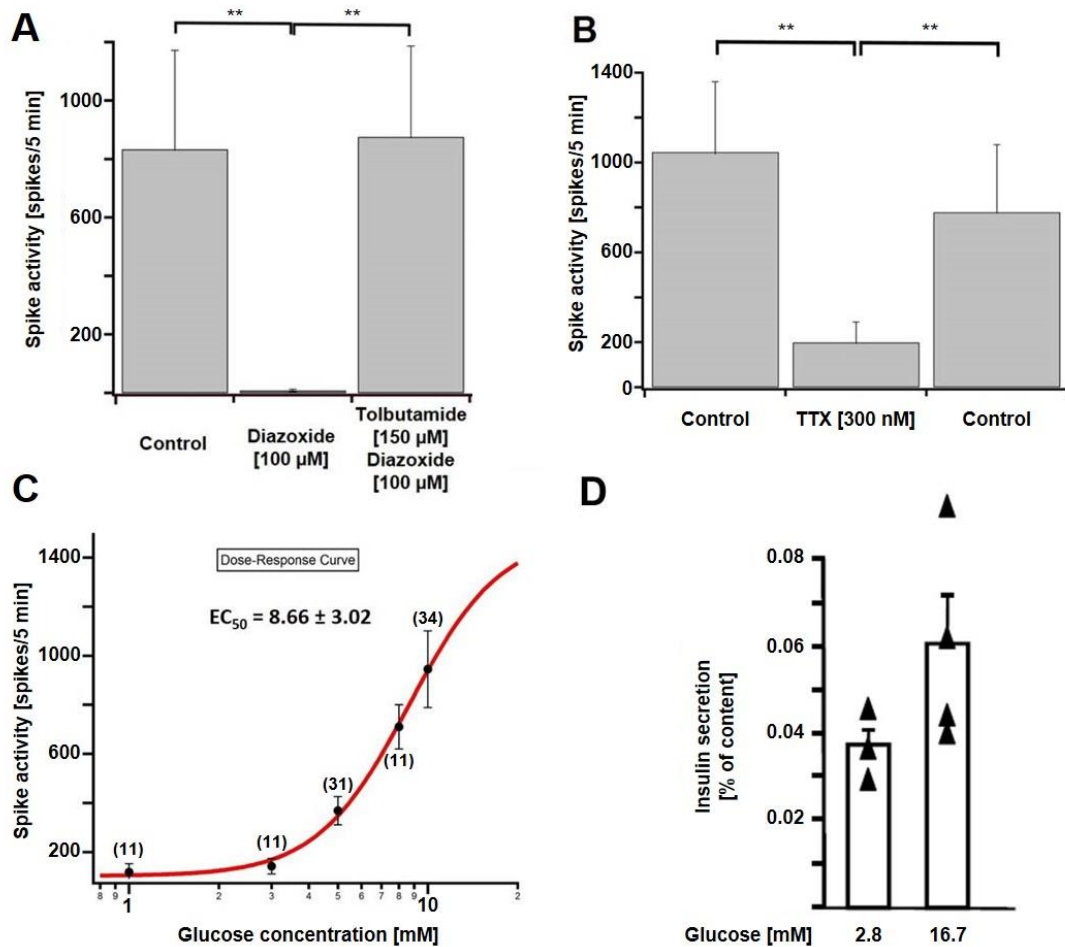
Fig.11: Recordings of human islets with the BetaScreen device and insulin secre-tion measurements A Effect of diazoxide and tolbutamide on spike activity of intact human islets. Diazoxide (100 µM, n=10) strongly reduced spike activity, whereas addi-tional application of tolbutamide (150 µM, n=10) reversed this inhibition. **B** TTX reduced spike activity significantly (300 nM, n=11). The effect of TTX is reversible, which can be seen after the washout of the toxin (n=11). **C** Glucose concentration-response curve of spike activity. Spike activity increased with increasing glucose concentrations. The data are fitted with the Hill equation and revealed a half-maximal glucose concentration of 8.66 ± 3.02 mM. **D** The insulin secretion experiment was performed from the workgroup of Prof. Dr. Susanne Ullrich from the Universitätsklinikum Tübingen and showed that the islets secreted only a small percentage of the stored insulin and glucose induced insulin secretion did not become significant indicating a low responsiveness of this preparation to glucose.

3   BetaScreen device

The aim of the project was to develop a MEA-based parallelized recording system for the investigation of acute intact islets of Langerhans called BetaScreen. The BetaScreen project was a cooperation between the company MCS (Multi Channel Systems, Reutlingen, Germany) and the institution NMI (Natural and Medical Science Institute at the University of Tübingen, Reutingen, Germany) under scientific consulting of Prof. Dr. G. Drews and Prof. Dr. P. Krippeit-Drews (Department of Pharmacology, Institute of Pharmacy, University of Tübingen, Germany). The development of the BetaScreen Device was funded by the BMBF-program KMU innovative: Biotechnolgie-BioChance, #0316162B.

3.1   Development of a MEA based parallelized screening system for primary intact islets of Langerhans called BetaScreen

Recordings of cultured islets (see chapter 2.3) is a very useful tool for long-term investigations of islets but not for acute drug application. This is due to the fact that the cultivation process, i.e. the attachment of the islets on the MEA surface, takes at least two days until the recordings can start. And the number of experiments for acute islet recordings so far was restricted to one recording per experiment which is too time consuming and cost-intensive due to high labor costs. Therefore, it was of great importance to find a way to enhance the throughput and the handling of the recordings to a convenient level to meet the need of both academic and industrial laboratories. The field of application for the BetaScreen device can comprise 1) clinical basic research in the field of experimental diabetes research 2) quality check of islets used for transplantation and 3) pre-clinical drug development.

3.2   First prototype of the BetaScreen recording device

The first recording device used for the BetaScreen project was based on the USB-MEA32STIM4-System from MCS with integrated amplifier, filter amplification and analog/digital board (Fig.12A). Moreover, the device was equipped with a perfusion heating and the possibility to apply suction (negative pressure) from below via a vacuum pump with pressure sensor and waste bottle (Fig.12C). For

the positioning of the islets via suction a new MEA development was necessary (Fig.12B). The first MEA prototype was equipped with five electrodes within a round recording chamber. In the middle of each electrode was a positioning hole
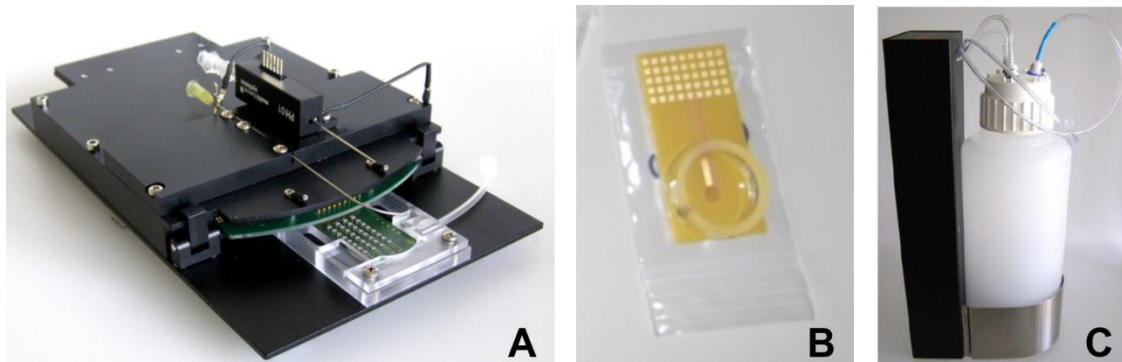
Fig.12: The first prototype of the BetaScreen device. **A** USB-MEA32STIM4 head-stage from MCS with integrated amplifier, filter amplification and analog/digital board. **B** Newly designed first MEA prototype with 5 electrodes within a round recording chamber (NMI). **C** Constant vacuum pump (CVP) with pressure sensor to record the target pressure in relation to the current ambient pressure.

arranged to enable the placement of islets via suction. Recordings with the new developed electrodes were not successful (n > 50). The initial presumption that the impedances of the electrodes are too high couldn't be confirmed. Impedance measurements revealed values of ≤ 50 kΩ (under a physiological relevant frequency of 1 kHz) which is in the optimal range (n=95). However, the examination of the hole structure showed that the main reason for the failure to record electrical activity was the fact that the contact of the islets to the electrodes was not tight enough. The reason for that were process-related differences in the structure of the holes which led to different heights of the hole rims on the MEA surface. This could be further confirmed by measurements of the diameter of the positioning holes which showed big differences. The diameter varied from 9 μm up to 32 μm (n=9) (see Fig.13).
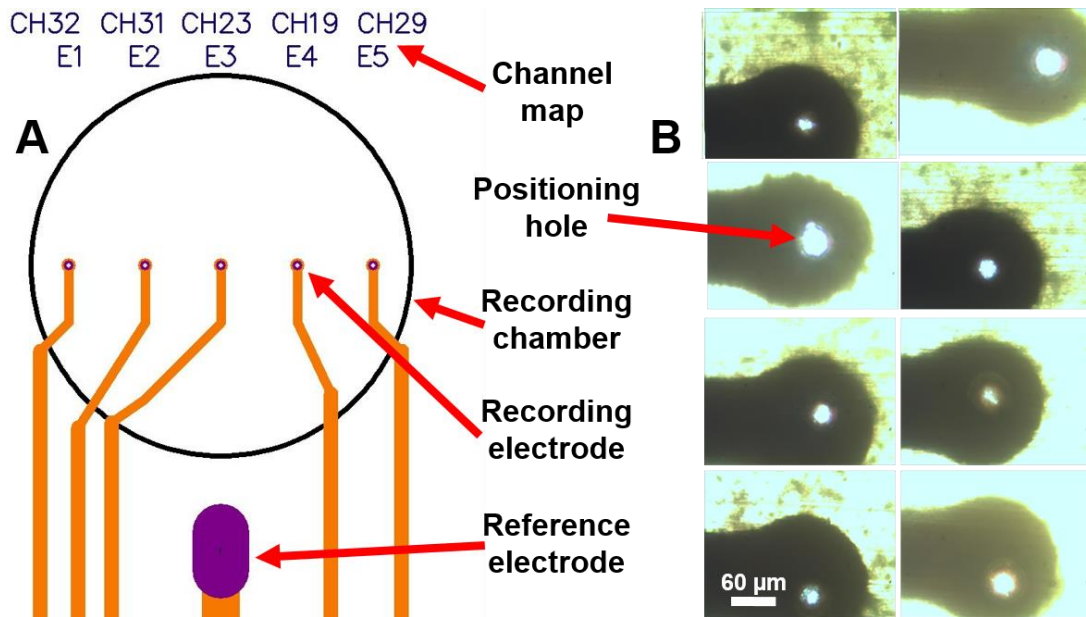
Fig.13: Technical drawing of the first MEA prototype for the BetaScreen device and microscopic photos of single electrodes with positioning holes. A Technical drawing of the MEA with channel map, round recording chamber, five recording electrodes and reference electrode (see red arrows). **B** Microscopic photos of eight recording electrodes with positioning holes (see red arrow). The differences in size and shape with irregular hole rims are clearly visible. The measurement of the diameter of the electrodes revealed big variations. The diameter varied from 9 µm up to 32 µm (n=9).

3.3   The usage of perforated MEAs for the placement and recordings of islets via suction

As it turned out that the first approach with the positioning hole in the centre of the electrodes did not provide a proper contact of the islets to the electrodes, a new strategy was needed. The new strategy was based on the idea of the already existing perforated MEA (pMEA) technology developed from MCS. These MEAs were developed to record electrical activity from acute brain slices placed via suction. The electrodes are placed on top of a polyimide foil and the foil is fixed to a ceramic substrate to achieve mechanical stability. The area around the recording electrodes is perforated which allows to either perfuse the biological probe from both sides or to apply negative pressure. For the first pilot studies I used modified pMEAs to test whether this approach can improve the contact of the islets to the electrodes. For the pilot study a polyimide foil with an exact defined hole was glued on top of the surface of the pMEAs, so that only three electrodes with positioning holes around were uncovered (see Fig.14). An additional improvement was the usage of titanium nitride electrodes which provide a better

signal to noise ratio due to lower impedances in comparison to the gold electrodes used for the first prototype.
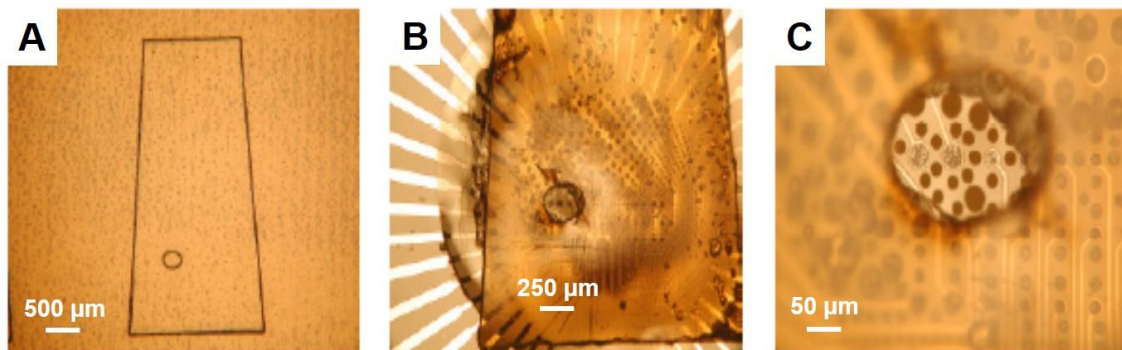
Fig.14: Detailed view of a pMEA with glued polyimide foil used for pilot study recordings. A Polyimide foil with defined hole at the lower left part of the foil. **B** pMEA with glued polyimide foil on top of the MEA surface. **C** Detailed view of the defined hole in the foil with recording electrodes and positioning holes in the center.

The attempt with the pMEAs worked and I recorded for the first time electrical activity from an islet in 10 mM glucose placed via suction on top of a MEA (see Fig.15). This was the "proof of principle" that this approach is possible and that islets are placeable and recordable by means of positioning holes and negative pressure. This first result showed that this strategy is promising and was the kickoff for a new pMEA-based development specifically designed for islet recordings.
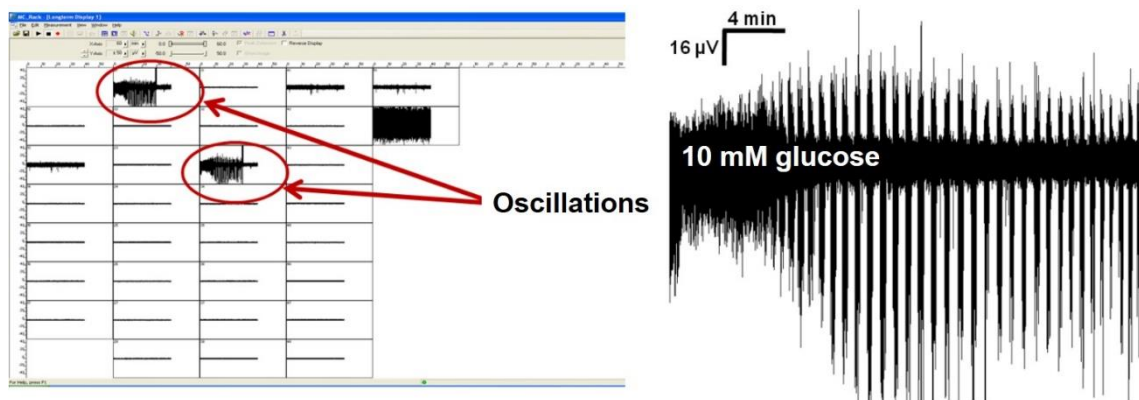
Fig.15: Proof of principle recording on top of a perforated MEA in 10 mM glucose. (Left) General view of oscillatory activity recorded from one islet on top of two electrodes **(Right)** Enlarged view of a trace with oscillations recorded with one of the electrodes on the left.

3.4 Development of new electrodes designed for the placement of islets via suction

As with the previously used prototypes the electrodes are placed on top of a polyimide foil. The electrode material is titanium nitride and negative pressure can be applied from the bottom side of the MEA. The electrodes are surrounded with positioning holes to ensure a proper positioning of the islets on top of the electrodes (see Fig.16). In order to find out which electrode size is optimal for the placement of islets two different electrode sizes were used, electrodes with 30 µm and with 50 µm in diameter (Fig.17).
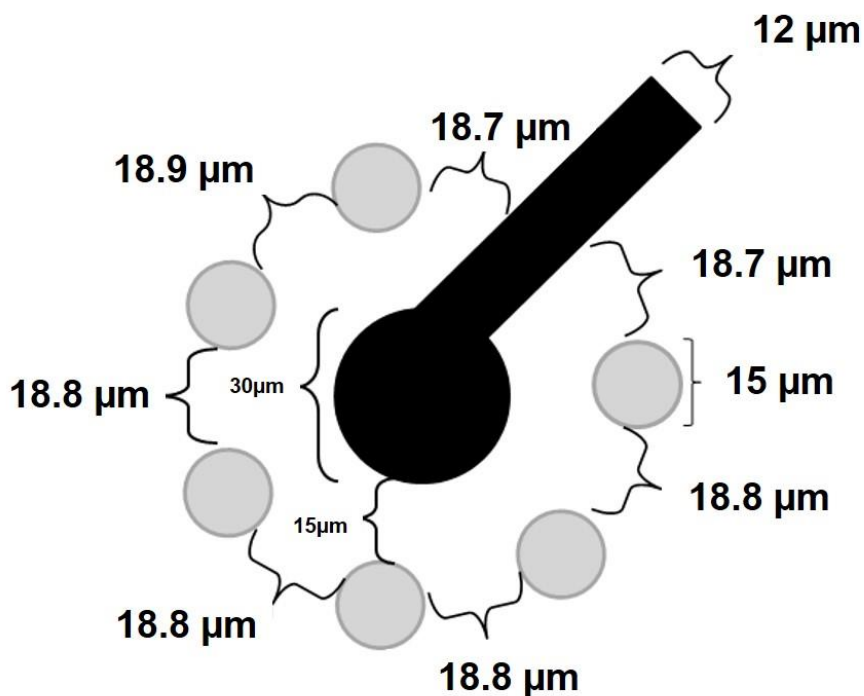
Fig.16: Draft of a new design of a 30 µm titanium nitride electrode. (Black) Recording electrode with 30 µm diameter (round shape) and electrode tracks 12 µm broad. **(Grey)** Positioning holes with 15 µm diameter are located around the recording electrode.

The quality measurements after the manufacturing process revealed impedances (at a frequency of 1 kHz values of ≤ 50 kΩ) of the electrodes which are comparable to the standard MEAs produced at the NMI and sold by MCS. The first MEAs used for the test recordings were equipped with five electrodes within a recording chamber. Negative pressure was applied with a vacuum pump and recording solution was perfused with a peristaltic perfusion system with one inlet and one outlet pump.
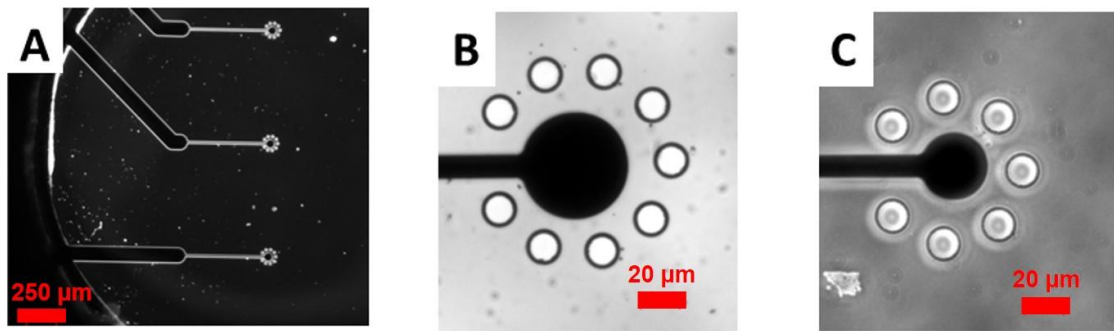
Fig.17: Microscopic image of the new electrodes. A Three electrodes located in a recording chamber with electrode tracks coming from the right **B** Electrode with 50 µm in diameter and surrounding positioning holes **C** Electrode with 30 µm and surrounding positioning holes.

One of the first recordings of electrical activity with the new electrodes is shown in figure 18. After pipetting the islets into the recording chamber they positioned themselves on top of the electrodes without further support. The application of 50 mbar negative pressure during the pipetting turned out to be ideal for the positioning. To avoid damage of the islets negative pressure was reduced to 10 mbar after the positioning process. Moreover, not only the placement could be facilitated, but even the signal to noise level is significantly improved in comparison to the acute recordings. The improved signal to noise ratio could be achieved due to a better contact between islets and electrodes. A further advantage is that the value of the negative pressure can be adjusted during an ongoing experiment to a currently needed value.
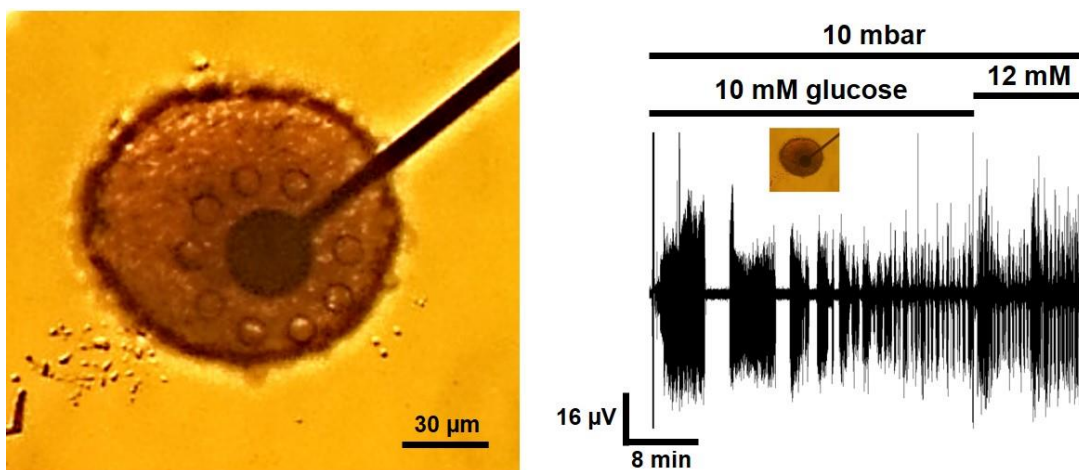
Fig.18: Proof of principle recording with a new designed BetaScreen electrode. (Left) Microscopic photo of an islet placed with 50 mbar negative pressure on top of a BetaScreen electrode which is surrounded with positioning holes. **(Right)** Trace with electrical activity of the islet which is shown on the left side in 10 and 12 mM glucose. Note that the negative pressure was reduced to 10 mbar after the placement of the islet. The signal to noise ratio is improved in comparison to acute recordings.

3.5   Physiological and pharmacological validation of the prototype system

An important question was whether the properties of the electrical activity are comparable to literature as well as to the acute recordings obtained with the classical MEA chips. To be sure that the positioning with negative pressure did not influence the behaviour of the recorded electrical signals, validation studies were necessary. The glucose concentration response curve plotted as FOPP (see Fig19A) recorded with the BetaScreen prototype revealed a half-maximal activity of 12,26 ± 2 mM, which is comparable to the results obtained with acute MEA recordings (12 mM) (Pfeiffer et al., 2011). Moreover the data are in agreement with data recorded with intracellular microelectrodes (11 mM) (Henquin, 2009). The values range from 0% without activity for 3 mM glucose (n=16) and the values increased with 6, 8, 10, 12 and 15 mM to 1 ± 1% (n=4), 27 ± 2% (n=12), 36 ± 4% (n=11), 46 ± 5% (n=8) and 65 ± 13% (n=6), respectively. To further validate the reproducibility of the new prototype the K_{ATP} channel modulators tolbutamide and diazoxide were tested (see Fig.19B). The application of 150 μM diazoxide inhibited oscillatory activity completely (n=13) whereas the additional application of 300 μM tolbutamide restored electrical activity (n=13) (Fig.19B). The results clearly demonstrated that the BetaScreen prototype electrode revealed the same electrophysiological results as the previously used standard MEAs. Further, the results are qualitatively in line with the results obtained with other electrophysiological recording techniques such as e.g. intracellular microelectrode recordings.
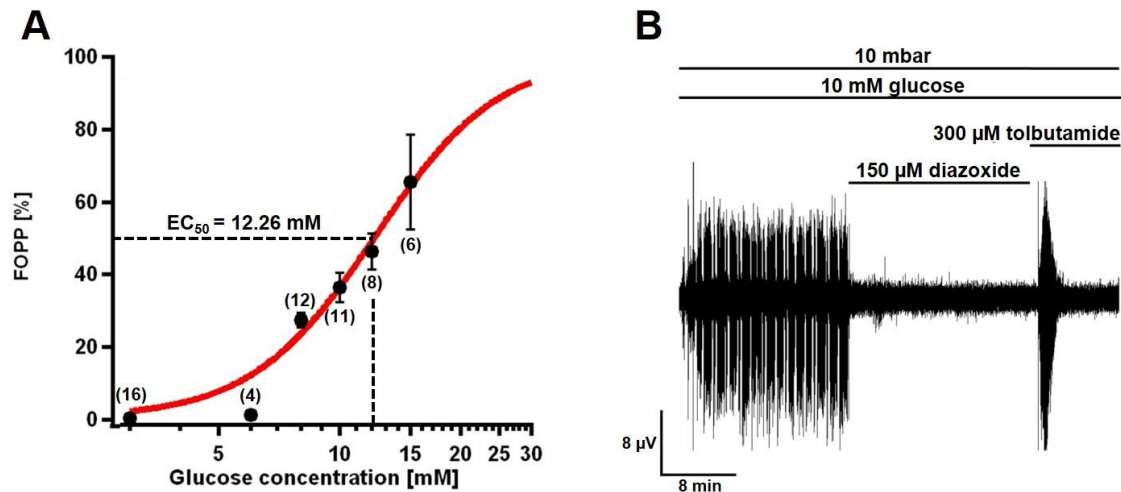
Fig.19: Glucose concentration response curve and pharmacological modulation of electrical activity with the K_{ATP} channel modulators diazoxide and tolbutamide. A Glucose concentration response curve plotted as FOPP [%] against glucose concentration [mM] recorded with the BetaScreen prototype displays a similar electrophysiological result as recorded with the standard MEAs. The half-maximal activity of 12.26 ± 2 mM is comparable and the shape of the concentration curve has the expected sigmoidal shape **B** Recording of electrical activity in 10 mM glucose (FOPP in % = 83) with a negative positioning pressure of 10 mbar. The application of 150 µM of the K_{ATP} channel modulator diazoxide (n=13) inhibit electrical activity completely (FOPP in 5 = 0), whereas an additional application of 300 µM tolbutamide transiently restored the activity (FOPP in % = n/a).

3.6   Expansion of the BetaScreen device to five recording chambers to increase the throughput of the recordings

The next important step was to increase the throughput of the BetaScreen device to enable parallel recordings. Thus a complete redesign of the previously used MEA was necessary. The new designed chip consists of five recording chambers with five electrodes in each chamber (see Fig.20). The new layout of the MEA enables to record electrical activity from 25 intact islets simultaneously. The new MEA chips consist of a pottery substrate which has five holes with a diameter of 5 mm. The holes are covered with a polyimide foil. The recording electrodes with the positioning holes are placed on the polyimide foil, so that each of the five holes contains five recording electrodes (see Fig.20).

On top of the pottery substrate (MEA surface) is a so called multiwell cover arranged (see Fig.21). The multiwell cover with his five recording chambers is applied with glue on top of the MEA. Each of the five chambers can be perfused independently. For a proper and leak protected placement of the MEA into the
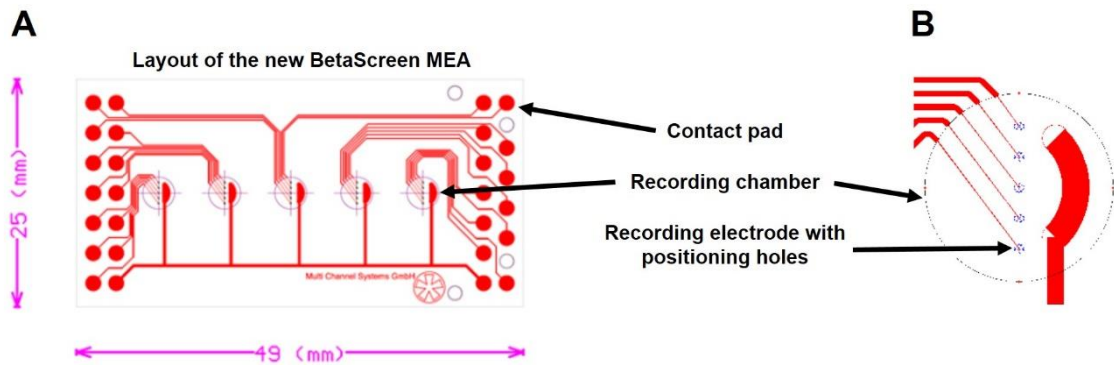
Fig.20: Schematic figure of the new BetaScreen MEA layout. A Total view of the BetaScreen MEA with contact pads and five recording chambers (see black arrows) **B** Magnification of a recordings chamber with five recording electrodes surrounded by positioning holes (see black arrows).

headstage an additional substrate is glued onto the bottom of the MEA (see Fig. 21). Moreover, the bottom substrate contains the connection for the perfusion system (influx and efflux) and as well the connection to the vacuum pump for the generation of suction. Figure 21Bc and d shows the fitting of the bottom substrate pins into small holes with integrated seals located in the headstage.
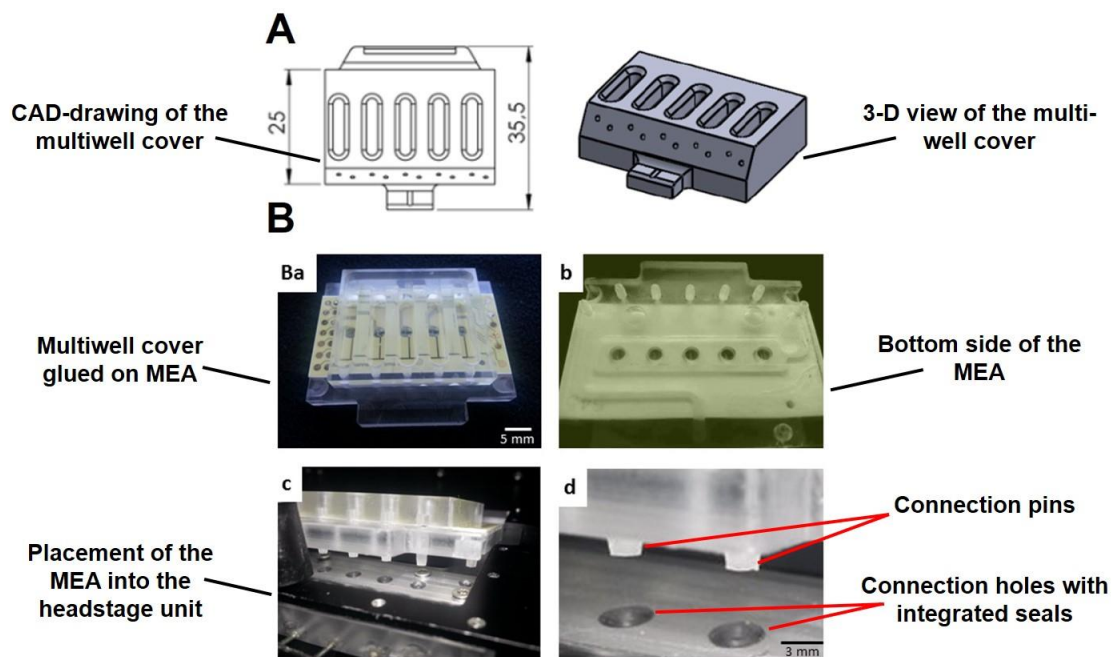
Fig.21: Layout of the multiwell cover, assembling of the MEA with the multiwell cover and the integration into the amplifier unit. A CAD-drawing of the multiwell cover. (Left) Top view of the multiwell cover with the five oval recording chambers in the centre of the drawing. (Right) 3-D view of the cover unit **Ba** The multiwell cover with five recordings chambers and the bottom substrate **(b)** is glued on both sides of the MEA. The five chambers can be perfused independently. Influx and efflux of the perfusion system and the connection to the vacuum pump is integrated. **Bc and d** The connection

between the MEA and the headstage unit is obtained via pins of the bottom substrate which stick into small holes located on the headstage. Leakage protection is gained with integrated seals.

Figure 22 shows the BetaScreen in use during an experiment. The microscopic photos shows the five independent recording chambers numbered from 1 till 5. The electrodes in recording chamber 2 till 5 are covered with islets. A screenshot of the recording software (MC_Rack, Multi Channel Systems, Reutingen) displays the measurements. The red-rimmed rectangles mark the corresponding electrodes of each recording chamber. Electrical activity was induced by 10 mM glucose and 12 islets displayed oscillatory activity. The new developed five chamber BetaScreen MEA enabled to record statistically signals from 4.6 ± 0.33 (n=32) chambers per recording. Within a single chamber the success rate reached an average of 2.9 ± 0.24 (n=57) islets per recording. At this stage of the BetaScreen development the throughput of the recordings could be increased by a factor of 13.34 in comparison to the previous used acute recording technique.
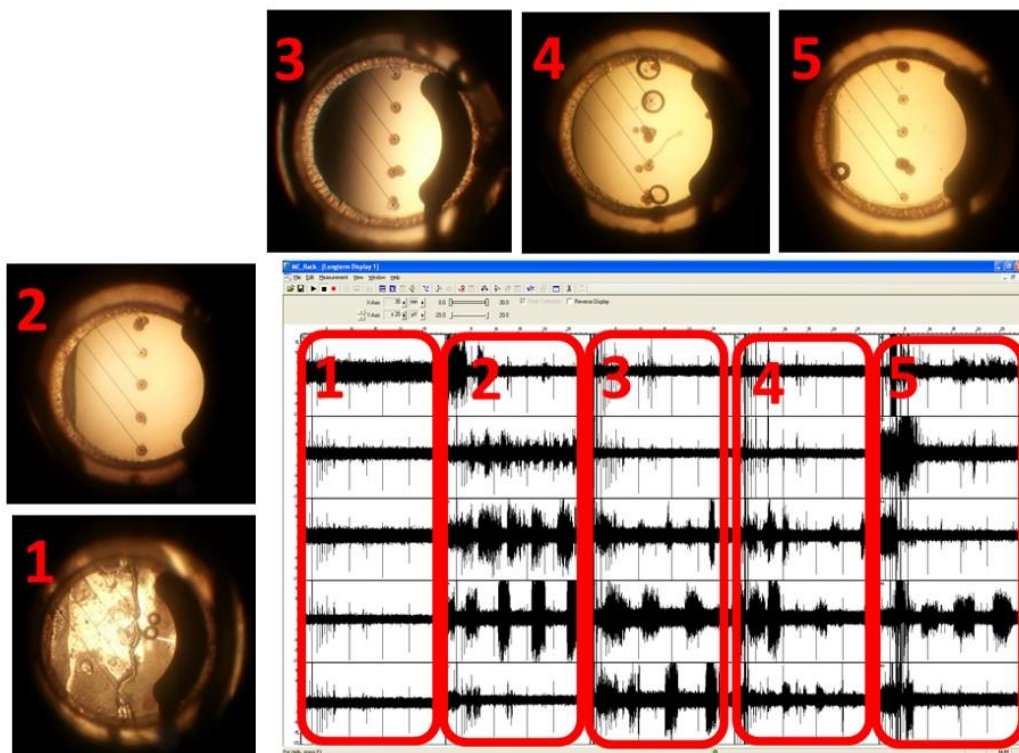
Fig. 22: Exemplary experiment with the newly developed BetaScreen device. The photos are showing the five recording chambers (1 to 5). In chamber 2 till 5 the electrodes are covered with islets. The screenshot shows the recording software and the red-

rimmed rectangles are displaying the corresponding electrodes of each chamber. Electrical activity was induced by 10 mM glucose and 12 islets displayed oscillatory activity. Note, the current layout of the BetaScreen allows to record electrical oscillations from up to 25 intact islets simultaneously.

In conclusion, this study shows the successful development of a parallelised MEA chip, which allows to record simultaneously electrical oscillations from up to 25 intact islets of Langerhans. The multi well cover on top of the new chip consists of five recording chambers, each chamber contains five electrodes. Each of the five chambers can be perfused independently. During the last phase of the project a success rate of almost 54% was reached, i.e. an average of 13.34 islet recordings per experiment. This is an improvement of throughput by a factor of 13.34 compared to the acute recordings at the beginning of the project. The validation of the BetaScreen device revealed positive results. The properties of the electrical activity are comparable to literature as well as to the acute recordings obtained with the classical MEA chips.

The BetaScreen device is an innovative new method which will facilitate the development of new pharmacological substances in the field of diabetes research based on electrical activity. Previous attempts to use electrophysiological features as readout for a higher throughput screening failed because of technical limitations, e.g. the invasiveness of the methods and the need of high technical skills. The BetaScreen device enables for the first time acute electrophysiological medium throughput recordings of whole intact islets. Moreover, it will facilitate basic research, e.g. in combination with knockout mice. Importantly, the capability to record also human islets (see chapter 2.4) opens numerous new possibilities for this approach, e.g. the system could be used as a quality control system prior to transplantation of human islets into patients with type-1-diabetes.

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5   Appendix

5.1   Danksagung

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5.2   Publications

Integrative Biology

PAPER

Long-term culture and functionality of pancreatic islets monitored using microelectrode arrays

Sven Schönecker,[a] Udo Kraushaar,[a] Martina Düfer,[b] Anika Sahr,[c] Carmen Härdtner,[c] Elke Guenther,[a] Reinhard Walther,[c] Uwe Lendeckel,[c] Winfried Barthlen,[d] Peter Krippeit-Drews[e] and Gisela Drews*[e]

Extracellular recording of the glucose-induced electrical activity of mouse islets of Langerhans on microelectrode arrays (MEAs) is an innovative and powerful tool to address beta-cell (patho-)physiology. In a dual approach we tested whether this technique can detect concentration-dependent drug effects as well as characterize alterations in beta-cell activity during prolonged culture. First we established conditions that allow long-term investigation of beta-cell function by recording electrical activity. The results provide the first measurements of beta-cell membrane potential oscillations of individual murine islets during long-term culture. Oscillations were recorded for up to 34 days after islet isolation. Importantly, the glucose dependence of electrical activity did not change over a period of one month. Thus we can follow electrophysiological changes of individual islets induced by alterations in the beta-cell environment over weeks. Second, we used the MEA technique to assay beta-cell damage induced by oxidative stress and to evaluate appropriate protection mechanisms. Oxidative stress plays a key role in the development of type 2 diabetes mellitus (T2DM). Examination of the acute effects of H_2O_2 on electrical activity showed that the oxidant reduced the electrical activity in a concentration-dependent manner. The superoxide dismutase mimetic, tempol, protected against the detrimental effects of H_2O_2. In conclusion, we demonstrated that MEA recordings can be used to address disease-related mechanisms and protective interventions in beta-cells. In the future, this fundamental work should enable the monitoring of the electrical activity of islets of Langerhans under controlled *ex vivo* conditions including long-term exposure to oxidative stress, glucolipotoxicity, and other diabetes-inducing agents.

Insight, innovation, integration

Type-2 diabetes mellitus (T2DM) is a tremendous health problem worldwide. Our work demonstrates that the microelectrode array (MEA) technique is an excellent tool to study *in vitro* the molecular basis of functional changes in beta-cells occurring during the development of type 2 diabetes mellitus. First, we established an *in vitro* model that allows long-term investigation of beta-cell function by registration of the electrical activity of isolated islets using the MEA technique. In the second step we applied oxidative stress to the islets which is crucial in the development of T2DM and evaluated appropriate protection mechanisms. This is a fundamental study that enables monitoring the electrical activity of murine islets in controlled *ex vivo* situations during long-term exposure to defined diabetes-associated, *e.g.* oxidative stress, glucolipotoxicity, and diabetes-inducing agents.

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Introduction

Insulin secretion of pancreatic beta-cells is determined by the degree of electrical activity, *i.e.* phases of depolarized membrane potential with action potentials and repolarized interbursts. Recently, we succeeded in recording the electrical activity of isolated mouse islets of Langerhans using planar, extracellular electrodes arranged in a microelectrode array (MEA).[1] This approach was confirmed by Raoux and coworkers.[2] The MEA system allows quantifying the electrical activity of beta-cells by calculating the fraction of the plateau phase (FOPP, the percentage of time with spike activity).

We demonstrated that the glucose dependence of the FOPP measured using a MEA equals that measured using sharp intracellular microelectrodes and is almost identical with the glucose dependence of insulin secretion measured with isolated mouse islets.[3] In addition, the MEA technology enables detection of the first phase of electrical activity elicited by an increase in glucose from a sub-stimulatory to a stimulatory concentration and allows simultaneous measurement of fluctuations in electrical activity and cytosolic Ca^{2+} concentration.[1]

These results convincingly demonstrate that MEAs are excellent and unique tools for reliable measurements of the FOPP. MEAs are superior to conventional electrophysiological methods since they are (1) non-invasive, (2) use whole islets that – in contrast to single cells – consistently display oscillations of electrical activity, and (3) easy to handle, thus increasing the experimental throughput enormously.

In humans, T2DM develops over many years. Current *in vitro* models to investigate alterations in the oscillatory activity of pancreatic islets seldom exceed a time period of several hours and are thus inadequate to study long-term changes. We demonstrate, for the first time, that the MEA approach allows monitoring of electrical activity and glucose-responsiveness of cultivated islets over a period of weeks. Increased oxidative stress within beta-cells due to excessive fuel intake or increased hormone levels (*e.g.* angiotensin II, endothelin) is a key event in the development of T2DM.[4] Since antioxidant defence mechanisms are low in beta-cells the characterization of strategies to prevent oxidative stress is a promising approach to identify beta-cell protective drugs. It is well known that oxidative stress reduces or even completely prevents glucose-mediated alterations in electrical activity,[5] thus the ability to make extended electrical measurements is attractive. We show that the MEA technology is very sensitive, allows detection of small changes in electrical activity caused by acute treatment of islets with an oxidant and can be used to determine the protective effect of a drug mimicking superoxide dismutase.

Taken together, we demonstrate the suitability of the MEA-based approach (1) to develop *in vitro* models for the study of long-term changes in islet function, (2) to investigate effects of diabetes-promoting conditions, *i.e.* oxidative stress, and (3) to test strategies for protection of islets against diabetes-associated functional cell damage.

Research design and methods

Islet preparation

Experiments were performed using intact islets of Langerhans isolated from adult C57Bl/6N mice (Janvier, France). The principles of laboratory animal care were followed according to German laws. Mice were euthanized by CO_2. Islets were isolated by collagenase digestion of the pancreas and cultured up to 34 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Solution and chemicals

Measurements of extracellular membrane potential were performed at 37 °C in a solution containing in mM: 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, pH 7.4, and glucose as indicated. The incubation medium for determining insulin secretion contained (in mM): 122 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.1 $MgCl_2$, 10 HEPES, pH 7.4. RPMI 1640, fetal calf serum, and penicillin/streptomycin were provided by Invitrogen (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

Recording setup for measuring membrane potential using extracellular electrodes

Extracellular membrane potential recordings were obtained with the MEA technique[6] using a MEA 1060-inv-standard amplifier system and software MC-Rack (Multi Channel Systems (MCS), Reutlingen, Germany). Data were low-pass filtered at 25 Hz and sampled at 1 kHz. Titanium-nitride electrodes had a diameter of 30 μm (200/30-Ti; MCS). In experiments with acute application of drugs one islet was placed on one of the 64 electrodes by means of a glass holding pipette with a tip angle of 30° (Reproline, Rheinbach, Germany) and a micromanipulator (Eppendorf, Hamburg, Germany). Extracellular voltage changes were only recorded from the electrode where the islet was placed using the grounded bath electrode as in ref. 1. In long-term culture experiments several islets were placed on different electrodes within the array.

Experimental procedure for measuring extracellular membrane potential

For acute drug application islets were used after 1–3 days of culture. Islets were incubated at 3 mM glucose for 10 to 20 min prior to experiments. Single islets were then transferred to the MEA bath chamber using a holding pipette and fixed to an electrode with gentle mechanical pressure. The islet was continuously perifused with bath solution throughout the experiment. Recordings always started in 3 mM glucose which gave a base-line with no electrical activity. Oscillatory activity was usually followed for 30–45 min for each condition and evaluated at periods of steady-state oscillations for the last 5–10 min before a new manoeuvre started. For long-term culture experiments the islets were kept in culture medium (RPMI, 11.1 mM glucose) on the MEA for the indicated time period. The culture medium was changed every second day. Each experiment started in culture medium before changing to the bath solution. For quantification of electrical activity the fraction of the plateau phase (FOPP) was determined by dividing a distinct time interval by the time with bursting activity within this distinct time interval.

Measurement of insulin secretion

After preparation islets were kept overnight in medium supplemented with 11.1 mM glucose. To determine insulin secretion, batches of 5 islets were incubated for 60 min at 37 °C with the indicated substances. Insulin was determined by radioimmunoassay using rat insulin (Crystal Chem. Inc., USA) as the standard.

Presentation of results

The recordings are representative of results from different islets; islets from at least 3 different mice were used for each series of experiments. Values are given as means ± SEM for the

indicated number of experiments. Statistical significance was assessed by ANOVA followed by the Student–Newman–Keuls post-test. A value of $p \leq 0.05$ was considered to be significant.

Results and discussion

Long-term measurements of beta-cell electrical activity

To study the effects of a diabetes-promoting environment, *e.g.* ROS, on the electrical activity of mouse beta-cells in more detail, it would be of great advantage to record changes over several days. However, all current electrophysiological techniques used in beta-cell research provide only short-term measurements of electrical activity, *i.e.* a range of minutes, or in exceptional cases, hours. In experiments with conventional microelectrodes or patch-clamp experiments the membrane potential measurement is regularly disturbed by leakage currents. Moreover, it is not possible to study the electrical activity of the same islet on different days. Therefore, we developed a system allowing long-term culture of isolated islets and, most importantly, regular measurements of the electrical activity of individual islets during culture. We validated physiological dynamics, *i.e.* the glucose responsiveness of the islets cultured on the MEA in the presence of 11.1 mM glucose and the appearance of oscillations over several weeks. As shown in Fig. 1A islets maintain their ability to respond adequately to glucose with oscillations up to 34 days. It was possible to record independent electrical activity simultaneously from several islets in contact with different electrodes on the MEA. Thus, the rate of successful experiments was increased compared to conventional electrophysiological methods.

As observed in acute experiments, at 3 mM glucose no oscillations were detected even after 34 days of culture. Differences in

the oscillations induced by 10 or 15 mM glucose, respectively, could be distinguished over time in the culture showing that the glucose dependence of electrical activity was preserved over several weeks (Fig. 1B). The data indicate that glucose metabolism is intact after long-term culture. The FOPP at 10 and 15 mM glucose, respectively, did not change over time. To evaluate possible culture time-dependent changes in electrical activity, the FOPP was summarized for experiments measured in the first week of culture (day 6 and 7) and compared with experiments performed in the last culture week (day 33 and 34). The FOPP amounted to $32 \pm 3\%$ ($n = 8$) and $36 \pm 2\%$ ($n = 7$) at 10 mM glucose and to $70 \pm 6\%$ ($n = 8$) and $72 \pm 5\%$ ($n = 7$) at 15 mM glucose at day 6/7 and day 33/34, respectively. The data clearly demonstrate that the FOPP at 10 and 15 mM glucose, respectively, did not change over time in culture. Notably, the number of non-responding islets is negligible with the MEA technology in contrast to microelectrode impaling. This paves the way to perform long-term experiments of electrical activity with low concentrations of redox-active compounds or diabetes-inducing agents and to study progressive changes evoked by gluco-, lipo- or glucolipotoxicity. Raoux and coworkers reported reduced action potential frequency of clonal INS-1E cells kept on a MEA after 3 days at elevated glucose concentration.[2] Moreover, the method permits co-culture of islets with other tissues to investigate their influence(s) on electrical activity.

In summary, the data presented show that MEA technology offers numerous novel and interesting long-term applications in beta-cell research, *e.g.* investigation of chronic effects of diabetes-inducing environments on electrical activity or the development of an *in vitro* model for testing short and long-term effects of potential antidiabetic drugs.

MEA technology detects protection against oxidative stress-induced pathophysiological changes of beta-cell electrical activity

Oxidative stress is an important pathogenic factor that contributes to the development of T2DM.[4a] We have shown that up-regulation of the antioxidant enzymes catalase (Cat), glutathione peroxidase (Gpx) and superoxide dismutase (SOD) in primary mouse beta-cells protects against apoptosis and loss of beta-cell function, respectively, that was induced by oxidative stress[5a] This is in agreement with observations of Lortz and co-workers who showed that overexpression of antioxidant enzymes reduces cytokine-induced cytotoxicity in insulin-secreting RINm5F cells.[7] H_2O_2 is one of the reactive oxygen species markedly contributing to oxidative stress in pancreatic islets. It is known to interact with several parameters of beta-cell stimulus-secretion coupling.[8] In the present study we investigated H_2O_2-evoked changes in electrical activity of mouse beta-cells and tested whether the membrane-permeable SOD mimetic, tempol,[9] protects beta-cells against H_2O_2-induced insult. First, we applied different concentrations of H_2O_2 in the presence of 10 mM glucose resulting in concentration-dependent inhibition of the FOPP that was well resolved by the MEA technology (Fig. 2A and B). Second, islets pre-incubated at 1 mM tempol prior to the
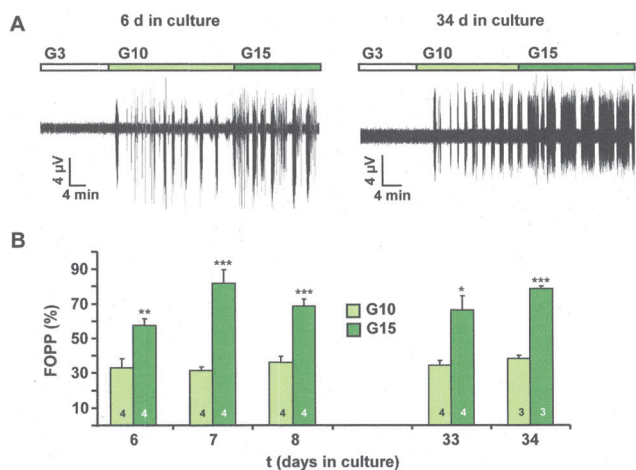
Fig. 1 Electrical activity of isolated mouse islets in long-term culture in the presence of 11.1 mM glucose. (A) Typical recordings showing electrical activity induced by 10 and 15 mM glucose and lack of activity in bath solution with 3 mM glucose after 6 and 34 days of culture. (B) Quantification of the FOPP in the presence of 10 and 15 mM glucose for different days of culture. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Statistical differences between the FOPP at 10 and 15 mM glucose at each culture day. The n-values within the columns indicate the number of islets. 5 different preparations are included in this series of experiments.
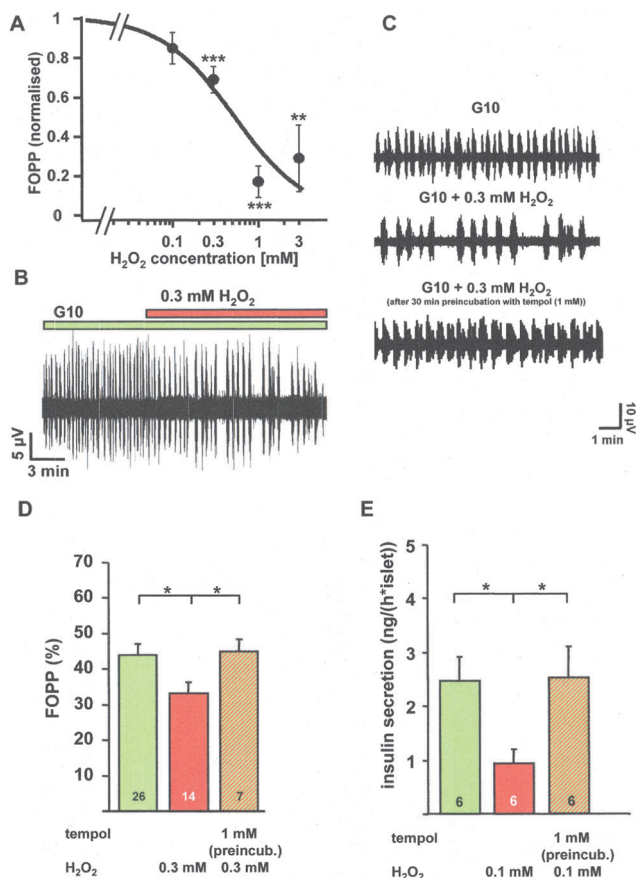
Fig. 2 Effect of H_2O_2 and the SOD mimetic tempol on insulin secretion and the electrical activity of mouse beta-cells. (A) Concentration–response curve of the effect of H_2O_2 on the fraction of the plateau phase (FOPP). $n = 3–14$. **$P \leq 0.01$, ***$P \leq 0.001$ compared to control without H_2O_2. (B) One typical recording out of 14 showing the application of 0.3 mM H_2O_2 in the presence of 10 mM glucose on electrical activity. (C) Original recordings in bath solution with 10 mM glucose (upper trace), 10 mM glucose and 0.3 mM H_2O_2 (middle trace) as well as in the presence of 10 mM glucose and 0.3 mM H_2O_2 after preincubation with 1 mM tempol (lower trace). (D) Quantification of the results presented as the FOPP. (E) Insulin secretion of islets *in vitro* in the presence of 15 mM glucose + 0.1 mM H_2O_2 + additional preincubation with 1 mM tempol.

addition of 300 μM H_2O_2 were compared to those exposed solely to 300 μM H_2O_2. To avoid direct interaction of tempol and H_2O_2, tempol was removed immediately before addition of H_2O_2. H_2O_2 significantly reduced the FOPP in the presence of 10 mM glucose but was without effect after 30 min of pre-incubation with 1 mM tempol (Fig. 2C and D). The SOD mimetic exerted a protective effect against the reduction of insulin secretion by 100 μM H_2O_2. In experiments without pre-treatment insulin secretion was reduced, but was unchanged after 15 min pre-incubation with the SOD mimetic (Fig. 2E). Tempol alone did not influence insulin secretion (2.91 ± 0.36 ng insulin per islet per h in the presence of 15 mM glucose *vs.* 3.18 ± 0.29 ng insulin per islet per h in the presence of 15 mM glucose and tempol; $n = 4$; n.s., not shown). The different efficacy of H_2O_2 on insulin secretion and electrical activity is most likely due to differences in diffusion in the two measurement

systems (1 h steady-state incubation for insulin secretion *vs.* perifusion for the measurement of electrical activity). The results clearly demonstrate that tempol preserves insulin secretion by circumventing the detrimental effect of H_2O_2 on stimulus-secretion-coupling. This agrees with our previous observations that up-regulation of antioxidant enzymes protects beta-cells against oxidative stress.[5a] The new data also show that the SOD plays a crucial role in the antioxidant defence of beta-cells and seems to be more important than Cat and Gpx since an SOD mimetic alone is sufficient to protect against an oxidative insult. At first glance it seems astonishing that an SOD mimetic protects electrical activity and insulin secretion against H_2O_2 since SOD does not degrade H_2O_2. It is assumed that tempol-dependent protection of the islets is due to detoxification of H_2O_2-induced ROS production including O_2^-. First, H_2O_2 leads to a partial depolarization of the mitochondria[5b] and a decrease of the mitochondrial membrane potential is assumed to increase ROS production. Second, ROS, including H_2O_2, can induce mitochondrial ROS release.[10] This mechanism has been extensively studied in cardiomyocytes and involves the mitochondrial permeability transition pore and/or the anion channel of the inner mitochondrial membrane. In both cases formation of superoxide anions in the mitochondria would rise and potentiate ROS generation and action. We suggest that this vicious circle is stopped by the SOD mimetic.

We have shown that a SOD mimetic protects beta-cells against acute oxidative stress pointing to the central role of SOD in antioxidant defence of pancreatic islets. Our experiments demonstrate that glucose-responsiveness of isolated islets cultured on MEAs is stable for up to 34 days. These data indicate that MEA technology is a valuable tool to investigate long-term effects of drugs influencing beta-cells *via* membrane potential-dependent pathways. The results suggest the feasibility of developing MEA-based *in vitro* disease models where islets are chronically challenged by glucolipotoxicity or stress-inducing agents thus enabling evaluation of intervention strategies.

Acknowledgements

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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Rapid functional evaluation of beta-cells by extracellular recording of membrane potential oscillations with microelectrode arrays

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Gisela Drews · Peter Krippeit-Drews**

Abstract The membrane potential (V_m) of beta-cells oscillates at glucose concentrations between ~6 and 25 mM, i.e. burst phases with action potentials alternate with silent interburst phases generating so-called slow waves. The slow waves drive oscillations of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and insulin secretion. The length of the bursts correlates with the amount of insulin release. Thus, the fraction of plateau phase (FOPP), i.e. the percentage of time with burst activity, is an excellent marker for beta-cell function and metabolic integrity. Extracellular voltage changes of mouse islets were measured using a microelectrode array (MEA) allowing the detection of burst and interburst phases. At a non-stimulating glucose concentration (3 mM) no electrical activity was detectable while bursting was continuous at 30 mM. The glucose concentration–response (determined as FOPP) curve revealed half-maximal stimulation at 12±1 mM (Hill equation fit). The signal was sensitive to K_{ATP} channel modulators, e.g. tolbutamide or diazoxide. Simultaneous recordings of electrical activity and $[Ca^{2+}]_c$ revealed congruent bursts and peaks, respectively. The extracellular recordings are in perfect agreement with more time-consuming intracellular electrical recordings. The results provide a 'proof-of-principle' for detection of beta-cell slow waves and determination of the FOPP using extracellular electrodes in a MEA-based system. The method is facile and provides the capability to study the effects of modulators of beta-cell function including possible anti-diabetic drugs in real time. Moreover, the method may be useful for checking the metabolic integrity of human donor islets prior to transplantation.

Keywords MEA · Extracellular recording · Islet · Slow waves · K_{ATP} channel · Insulin secretion

Introduction

The early work of Dean and Matthews [4] established that pancreatic beta-cells are electrically excitable. The beta-cell membrane potential (V_m) exhibits slow waves of electrical activity [7], consisting of bursts of Ca^{2+} action potentials and silent interburst phases. This fluctuating electrical activity actuates slow oscillations of $[Ca^{2+}]_c$ [18] and insulin secretion [2, 10]. The fraction of plateau phase (FOPP) is an excellent marker for intact beta-cell function and metabolism [7].

Before the introduction of the patch-clamp technique the determination of FOPP required measuring with sharp microelectrodes to elucidate electrical properties of beta-cells [7, 12, 13]. Both electrophysiological methods are technically demanding and are too time consuming to be used routinely. Moreover, it is difficult to detect V_m oscillations of single or small beta-cell clusters with patch-clamping [6, 11, 15] although slow waves are

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invariably detectable when intact islets with electrically coupled cells are impaled by intracellular microelectrodes [6]. To circumvent this problem Göpel and co-workers developed a technique to patch-clamp whole islets [11], but the method is technically challenging and time consuming. Different patterns of $[Ca^{2+}]_c$ oscillations between single cells and islets have been reported [9] emphasizing that measurement of oscillations from whole islets is more reliable and closer to the in vivo situation.

Microelectrode arrays (MEAs) present an alternative way to study the electrical activity of single cells or tissue slices and have been used successfully on cells from the heart, central and peripheral nervous system, and muscles [14, 20]. Data on the use of extracellular electrodes to record electrical activity from islets are scarce [3, 17] possibly because these attempts did not successfully resolve slow wave oscillations. Here we report on a non-invasive MEA-based system for the detection of glucose-induced slow waves in islets. This report is the 'proof-of-principle' for a method which can be extended for low-throughput to medium-throughput screening and thus allow the routine use of islets of Langerhans in industrial research. Additionally, the method is applicable to testing the metabolic status of human donor islets prior to transplantation.

Research design and methods

Islet preparation

Experiments were performed using intact islets of Langerhans isolated from adult C57Bl/6 N or NMRI mice (Janvier, France). The principles of laboratory animal care were followed according to German laws. Mice were euthanized by CO_2 application. Islets were isolated by collagenase digestion of the pancreas and cultured up to 3 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solution and chemicals

Measurements of $[Ca^{2+}]_c$, membrane potential changes by MEA (both 37°C), and patch-clamp recordings (34°C) were performed in a solution containing in millimolar: 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, pH 7.4, and glucose as indicated. Recordings of V_m with microelectrodes were done in a bath solution composed of (in millimolar): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 15 glucose, aerated with 95% O_2 and 5% CO_2 to maintain a pH of 7.4 at 37°C.

RPMI 1640, fetal calf serum, penicillin/streptomycin, and fura-2 were provided by Invitrogen (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Roth (Karlsruhe, Germany), or Merck (Darmstadt, Germany).
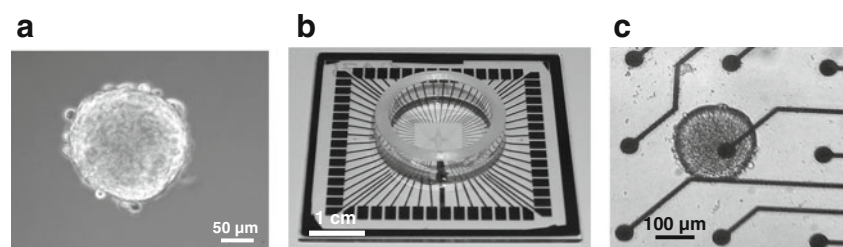
Recording setup

Extracellular membrane potential recordings were obtained with the microelectrode array (MEA) technique [20] using a MEA USB-1060 system with software MC-Rack (Multi Channel Systems (MCS), Reutlingen, Germany). Data were low-pass filtered at 70 or 100 Hz and sampled at 10 kHz. Titanium-nitride electrodes had a diameter of 30 μm (200/ 30-Ti; MCS). An islet was placed on one of the 59 electrodes by means of a glass holding pipette with a tip angle of 30° (Reproline, Rheinbach, Germany) and a micromanipulator (Eppendorf, Hamburg, Germany). Extracellular voltage changes were only recorded from the electrode where the islet was placed on (Fig. 1c) using the grounded bath electrode as reference.

Equipment and software for patch-clamp recordings and fluorescence measurements were from HEKA (Lambrecht, Germany) and from TILL photonics (Gräfelfing, Germany), respectively.

Electrophysiological experiments

Islets with a diameter of ~150 μm (Fig. 1a) were used for experiments after 1–3 days in culture. Islets were incubated in 3 mM glucose for at least 30 min prior to experiments. Single islets were then transferred to the MEA bath chamber (Fig. 1b, c), using a holding pipette with a negative pressure of about −15 mbar and fixed to an electrode with gentle mechanic pressure. The islet was continuously perifused with bath solution throughout the experiment. Recordings always started in 3 mM glucose

Fig. 1 a Microscopic view of a single murine islet of Langerhans. b A microelectrode array (MEA, Multi Channel Systems). c Islet of Langerhans placed on top of a MEA electrode

a
50 μm

b
1 cm

c
100 μm
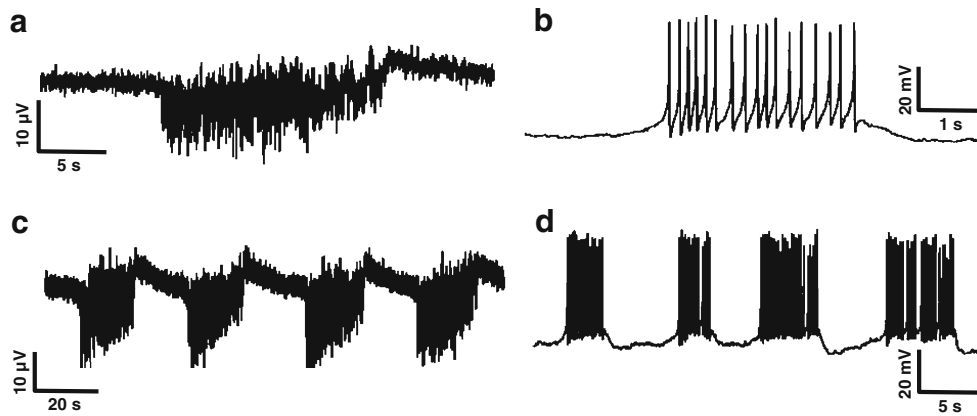
Fig. 2 Extracellular recordings with the MEA technique (**a**, **c**) and intracellular recordings obtained using the perforated-patch technique (**b**, **d**) at 15 mM glucose. **a**, **b** Single burst of APs of beta-cells in an islet. Note that the AP voltage deflections are negative or positive when measured extracellularly or intracellularly. **c**, **d** Four consecutive oscillations in 15 mM glucose. Representative recordings for 8 extracellular and 5 intracellular measurements, respectively

which gave a base-line with no electrical activity. Oscillatory activity was usually followed for 30–45 min for each condition and evaluated at periods of steady-state oscillations for the last 5–10 min before a new manoeuvre started.

Patch-clamp and intracellular microelectrode recordings were performed according to Düfer et al. [8]. $[Ca^{2+}]_c$ was determined as the ratio of fura-2 fluorescence at 360-nm and 380-nm excitation wavelength. The MEA was mounted on an inverted microscope (Zeiss, Stuttgart, Germany), and a pair of images was taken every 10 s. Because the glass of

the MEA absorbed too much light at 340 nm, 360 nm excitation was used. The large difference in the ratios of the $[Ca^{2+}]_c$ measurements between the experiments shown in Fig. 5 is presumably due to different absorption of light by the MEA since the islets were covered to a different extent by the electrode and/or conductor traces (cf. Fig. 1c).

Presentation of results

The recordings are representative of results from different islets; at least 3 different preparations were used for each

Fig. 3 Electrical activity induced in beta-cells by an increase in glucose concentration from 3 to 15 mM. One representative of eight extracellular recordings (**a**) is compared to one of three intracellular microelectrode recordings (**b**). Note that in both measurements the first phase with a long burst is followed by a longer interburst. The second phase consists of more or less regular oscillations. In **c** the effect of 100 μM diazoxide and the additional application of 150 μM tolbutamide (indicated by *horizontal bars*) on an extracellular recording of an islet in 15 mM glucose is shown. One of eight experiments with similar results
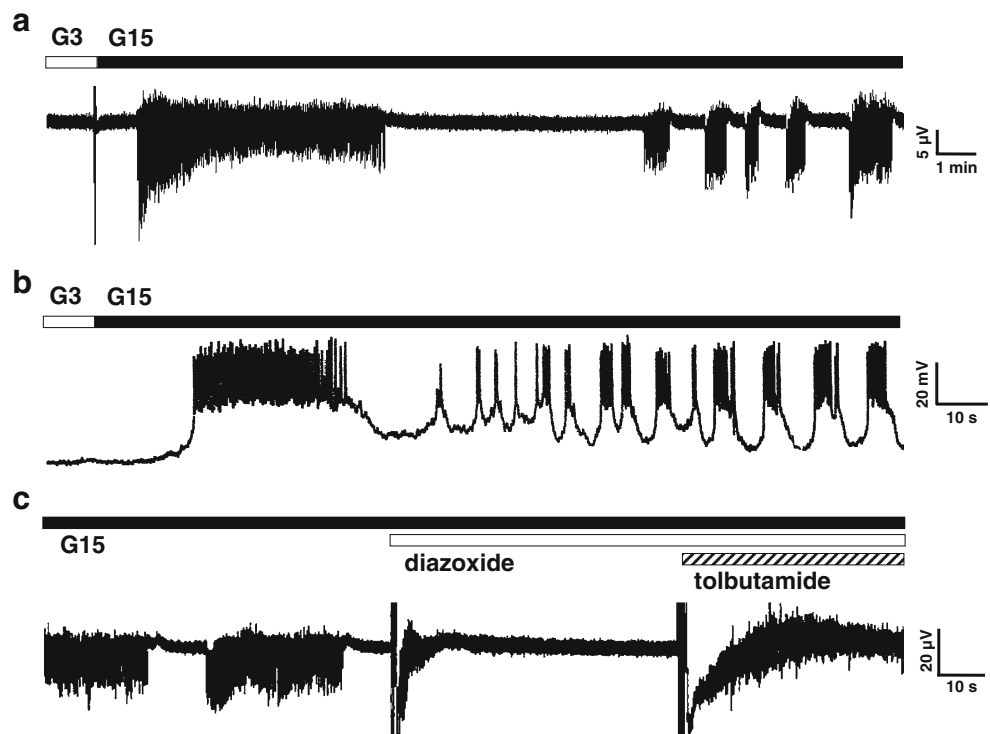
series of experiments. Values are given as means±SEM for the indicated number of experiments. Statistical significance was assessed by ANOVA followed by the Student–Newman–Keuls post-test. A value of $p \leq 0.05$ was considered to be significant.

Results

To investigate whether a MEA-based setup can reliably detect the electrical activity of intact islets of Langerhans, extracellular recordings were compared to intracellular measurements obtained by traditional techniques.

Figure 2a, c displays traces measured with the new MEA technique, while traces in Fig. 2b, d show intracellular recordings obtained with the perforated-patch technique. Figure 2a, b shows one burst of glucose-induced Ca^{2+} action potentials (APs) at an expanded time scale. Since the changes of V_m shown in Fig. 2a are measured by extracellular electrodes, the AP voltage deflection is negative with reference to the grounded bath electrode. Thus, this recording reflects to a first approximation the negative of the first derivative of APs measured with intracellular electrodes which are by definition positive voltage deflections (Fig. 2b).

Figure 2c, d shows that glucose-induced slow waves of mouse beta-cells exhibit similar patterns of electrical activity regardless of whether they are recorded by MEAs or intracellular techniques. Obviously the time course of the slow waves is different (note different time scales). This is presumably due to differences between individual islets. While the variation in individual burst and inter-burst times between different islets may be large, the relationship between both, i.e. the FOPP at a given glucose concentration, varies only in a narrow range. In addition, it may be that invasive electrodes induce a depolarising leakage current which makes the bursting faster. Figure 2c shows that the time resolution of extracellular recordings is sufficient to clearly discriminate burst and interburst phases and enable the determination and calculation of FOPP.

According to the consensus model of the stimulus-secretion coupling, electrical activity starts as soon as the ATP concentration close to the K_{ATP} channels is sufficiently increased to affect the channel activity [1, 19]. The glucose-induced ATP production of metabolically intact beta-cells leads to closure of K_{ATP} channels and as a consequence to the depolarization of V_m [1, 7, 12]. Typically, a biphasic rise of V_m with a first phase characterized by a very long burst followed by an extended silent period is observed. In the second phase the typical oscillations of V_m occur. Figure 3a, b illustrates that this characteristic pattern of electrical activity is obtained with both, extracellular (MEA) and intracellular (micro-
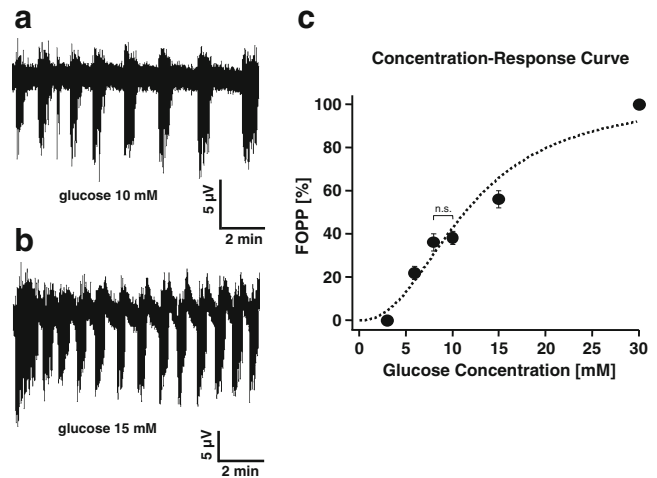
Fig. 4 **a**, **b** Representative MEA recordings from islets in 10 or 15 mM glucose. **c** Glucose concentration–FOPP curve measured with MEA electrodes. Data are means±SEM for 3 mM (n=10), 6 mM (n=5), 8 mM (n=5), 10 mM (n=8), 15 mM (n=11), and 30 mM (n=5) glucose, respectively. The data are fitted with the Hill equation (*dotted line*) giving a half-maximal glucose concentration of 12±1 mM. All values are significantly different from each other ($p \leq 0.05$) except those depicted as *n.s.*

electrodes) measurements. Again the burst duration in these examples is different, but a similar FOPP can be obtained in the second phase. Thus, the metabolic integrity of an islet can be estimated by extracellular measurements of changes in V_m.

Figure 3c shows the electrical activity (slow waves) of an islet in 15 mM glucose recorded with extracellular MEA electrodes which can be silenced by the addition of the K_{ATP} channel opener diazoxide (100 μM) and that APs reappear after addition of tolbutamide (150 μM).
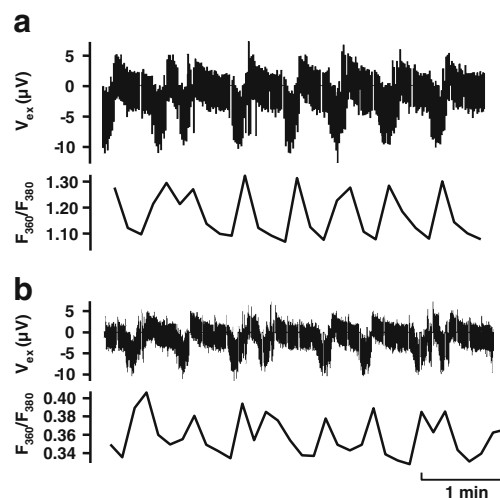
Fig. 5 **a**, **b** Two examples of simultaneous recordings of electrical activity and $[Ca^{2+}]_c$. The upper traces show glucose-induced electrical activity, and the lower traces are measurements of $[Ca^{2+}]_c$. Note that each burst of APs is accompanied by a peak in $[Ca^{2+}]_c$. The glucose concentration was 10 mM

Figure 4 shows the glucose dependency of the FOPP recorded with MEA electrodes. Traces in Fig. 4a, b show representative recordings in 10 mM and 15 mM glucose. The glucose concentration–response curve of the FOPP provides values of 0% (no activity) for 3 mM glucose ($n=10$) or 100% (continuous spiking) for 30 mM glucose ($n=5$). Consistent with values for intracellular recordings [12] the FOPP increased to $22\pm3\%$ ($n=5$), $36\pm4\%$ ($n=5$), $38\pm3\%$ ($n=8$), and $56\pm4\%$ ($n=11$) with 6, 8, 10, and 15 mM glucose, respectively. Fitting a Hill equation to these data (Fig. 4c) revealed half-maximal activity (50% FOPP) for 12 ± 1 mM glucose which is close to the value of 11 mM glucose measured with intracellular microelectrodes [12].

In 1991 Santos and co-workers [18] have shown that simultaneous measurements of V_m with intracellular electrodes and $[Ca^{2+}]_c$ exhibit synchronous oscillations. Figure 5 compares electrical measurements by extracellular recording with $[Ca^{2+}]_c$. Two examples are given (Fig. 5a, b) respectively; the upper traces show glucose-induced electrical activity, and the lower traces are measurements of $[Ca^{2+}]_c$. Each burst of action potentials is accompanied by a peak in $[Ca^{2+}]_c$.

Discussion

Pancreatic beta-cells are unique as they exhibit oscillatory electrical activity known as slow waves in response to glucose [7]. The FOPP rises proportional to the glucose concentration within a range between ~6 and 25 mM with a half-maximum response of approximately 11 mM [12]. At concentrations >25 mM continuous action potential firing occurs whereas at lower glucose concentrations (<6 mM) beta-cells are electrically silent [7]. Extracellular MEA recordings from whole islets exhibit the same glucose dependence (Fig. 3). Thus, possible contamination from the electrical activity of alpha-cells that exhibit the opposite glucose dependence, i.e. activation at low and inhibition at high glucose [7], is negligible. This can be explicitly seen in Fig. 3a where the glucose concentration was changed from 3 to 15 mM. If alpha-cell electrical activity contributed to the background noise seen in 3 mM glucose, it should be silenced when the glucose concentration was increased (just before beta-cells were activated), but no change was detected in this and similar experiments.

The detection of islet cell electrical activity by extracellular electrodes has been reported so far by two groups [3, 17]. To our knowledge this is the first demonstration of glucose-induced slow waves and the first calculation of the glucose dependence of FOPP using MEA technology.

As $[Ca^{2+}]_c$ [18] and importantly insulin secretion [2, 10] are coupled directly to the fraction of time beta-cells are bursting, FOPP provides an excellent marker for the actual insulin release from islets in response to glucose and/or test substances. Two examples (see Fig. 5) illustrate the close coupling between bursts measured with extracellular electrodes and $[Ca^{2+}]_c$. Every burst of action potentials produces a rise in $[Ca^{2+}]_c$ as previously shown for simultaneous measurements with microelectrodes and Ca^{2+} imaging [18]. Thus, determining FOPP with an extracellular microelectrode array provides a facile means to estimate insulin release in real time in response to various stimuli. Compared to patch-clamp or Ca^{2+} measurements by fluorescence techniques the method has the advantage to be non-invasive. Other types of cells and tissue slices have been maintained on MEAs for long periods; [14] thus, the method offers the possibility of recording from the same islet multiple times at intervals of hours or days. Therefore, MEAs allow recording of both acute changes of beta-cell electrical activity and the chronic effects of metabolites or drugs. MEAs should be useful for investigating co-cultures, e.g. how co-culturing pig islets with spleen cells affects insulin secretion [21] or how bone marrow increases the survival time of human islets [16]. MEAs could be used to study co-cultures of macrophages and islets and determine the action of macrophage-released substances on beta-cell electrical activity.

Finally, MEA technology can be applied to human islets. A screening system allowing the simultaneous measurement of oscillating insulin secretion by up to 15 single islets has been published recently [5], but the required manual encapsulation of single islets and the use of specifically labelled insulin, insulin antibody, and a means of fluorescence detection make the assay technically difficult.

We have provided a "proof-of-principle" that FOPP can be measured using single islets and extracellular electrode arrays. These arrays have multiple electrodes which can be recorded from independently; thus, it is feasible to develop low-throughput to medium-throughput assay systems where the electrical activity of 50 or more islets can be measured simultaneously. This would provide an ideal tool to evaluate the metabolic state of primary islets of Langerhans and to test the effects of metabolites, cytokines, drugs, and co-cultured cells.

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Human Islets Exhibit Electrical Activity on Microelectrode Arrays (MEA)

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Abstract

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This study demonstrates for the first time that the microelectrode array (MEA) technique allows analysis of electrical activity of islets isolated from human biopsies. We have shown before that this method, i.e., measuring beta cell electrical activity with extracellular electrodes, is a powerful tool to assess glucose responsiveness of isolated murine islets. In the present study, human islets were shown to exhibit glucose-dependent oscillatory electrical activity. The glucose responsiveness could be furthermore demonstrated by an increase of insulin secretion in response to glucose. Electrical activity was increased by tolbutamide and inhibited by diazoxide. In human islets bursts of electrical activity were markedly blunted by the Na^+ channel inhibitor tetrodotoxin which does not affect electrical activity in mouse islets. Thus, the MEA technique emerges as a powerful tool to decipher online the unique features of human islets. Additionally, this technique will enable research with human islets even if only a few islets are available and it will allow a fast and easy test of metabolic integrity of islets destined for transplantation.

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Introduction

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This study depicts a proof of principle that it is possible to measure oscillatory electrical activity of human islets with extracellular electrodes. The formation of glucose-induced oscillations of the plasma membrane potential V_m in beta-cells is a matter of interest for more than 25 years [1]. We have shown previously that recording of electrical activity with extracellular electrodes on micro-electrode arrays (MEA) is an excellent and easy-to-use tool to evaluate beta-cell function of mouse islets [2,3]. The percentage of time with burst activity, the so-called fraction of plateau phase (FOPP), is glucose-dependent and determines the amount of insulin secretion [1,4]. Metabolism-dependent closure of K_{ATP} channels and the subsequent membrane depolarisation, the so-called stimulus-secretion coupling of human beta-cells seems to be not very different to rodents [5]. Human beta-cells even exhibit a similar pattern of electrical activity, consisting of bursts of action potentials (APs) and silent interbursts [5], the so-called slow waves [1]. Although the initiation of the first burst of APs is similar in human and mouse beta-cells, the AP generation and burst termination may differ in some respects between the 2 species [5–8].

We show here that glucose-induced electrical activity of isolated human islets can be resolved and analysed with the MEA technique. We propose that the non-invasive extracellular recordings can be seminal for human beta-cell research as it is a valuable tool to assess on-line the function of human islets and may allow research with only a few islets at a time, e.g., when the number of islets in a biopsy is limited. This first demonstration presents only some occasional measurements and thus may be yet not more than a "case report", but it suggests that the method is suitable to study factors which modify membrane oscillations of human islets. Furthermore, this non-invasive, increased throughput method will be useful to on-line assess the responsiveness of single isolated human islets, e.g., prior to transplantation.

Methods

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Human islets were obtained from biopsies taken during pancreatic surgery according to the approvals of the ethics commissions of the Medical Faculty and the University Hospital of the University of Tübingen (No. 359/2013BO2) and the Universitätsmedizin Greifswald (BB 050/13).

The islets were isolated by injecting collagenase (2–4 mg/ml, Serva , Heidelberg, Germany) into the biopsy material and by handpicking after digestion at 37 °C. Islets from mice were isolated as described previously [2]. For measuring electrical activity of human islets in pancreas slices a biopsy was embedded in 4% low-melting agarose. Slices of 300 µm thickness were cut with a Leica VT1000 S vibrating blade microtome (Leica, Wetzlar, Germany) Islets and pancreatic slices were cultured overnight in RPMI 1640 (mice, 11 m M glucose) or in DMEM or CMRL1066 (human, 5 mM glucose). All media were supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. The principles of laboratory animal care were followed according to German laws.

Recordings of extracellular membrane potential were performed at 37 °C in a solution containing in mM: 140 NaCl, 5 KCl, 1.2 MgCl$_2$, 2.5 CaCl$_2$, 10 HEPES, pH 7.4, and glucose as indicated. All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).
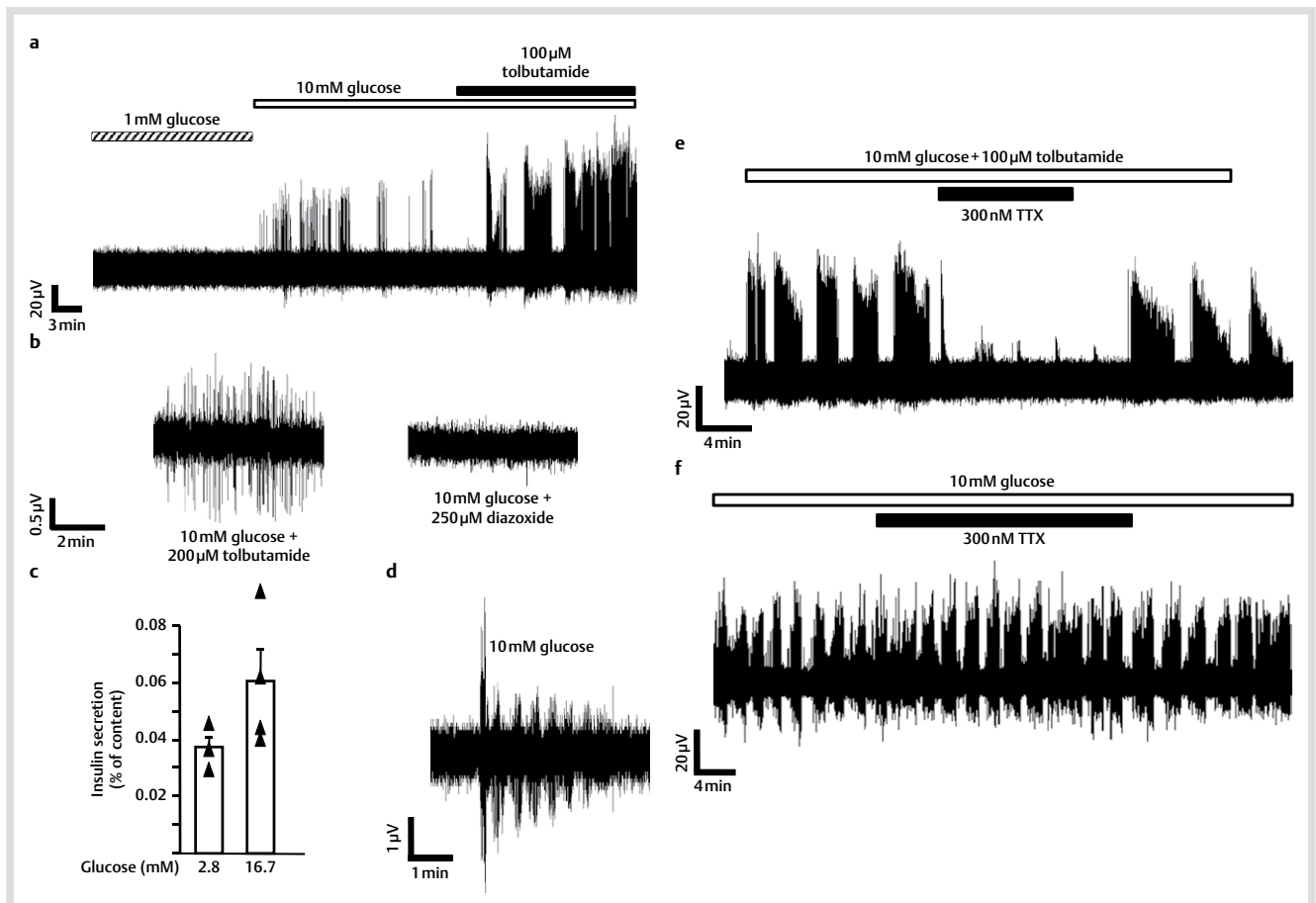
Extracellular membrane potential recordings were obtained with the MEA technique as described previously [2] using a MEA 1060-inv-standard amplifier system and MC-Rack software (Multi Channel Systems (MCS), Reutlingen, Germany). Isolated islets were placed on an individual electrode within an electrode array, and extracellular voltage changes were recorded by this electrode. For measuring electrical activity of human islets in pancreas slices they were placed on the MEA by means of a platinum grid. Islets or slices were continuously perifused with bath solution throughout the experiment.

For insulin secretion, batches of islets (10 islets/500 µl) were incubated in modified Krebs-Ringer-bicarbonate buffered saline as described previously and insulin was measured by radioimmunoassay [9].

Results and Discussion

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Extracellular recordings of isolated human islets are not depending on the age of the donor. ◉ Fig. 1 shows MEA recordings from an adult donor (◉ Fig. 1a, >60 years old) and from 2 children (◉ Fig. 1b, d, <1 year old), suffering from a pancreas tumour and congenital hyperinsulinism (CHI), respectively. The islet in ◉ Fig. 1a was electrically silent in 1 mM glucose and started bursting in 10 mM glucose. The sulfonylurea tolbutamide (100 µM) clearly increased the glucose-induced activity to almost continuous spiking (◉ Fig. 1a). The islet shown in ◉ Fig. 1b responded to tolbutamide (200 µM) in the presence of 10 mM glucose with spiking at low frequency, but spiking could be inhibited by diazoxide

Fig. 1   Electrical activity of human islets. **a** Electrical activity of an isolated human islet of 3 Langerhans received from an adult donor at different glucose concentrations and in 4 the presence of tolbutamide. **b** Electrical activity of an isolated human islet received 5 from an infant donor. Low frequency spiking of an islet during the application of 10 mM glucose and tolbutamide (left) and suppression of spike activity by diazoxide (right). **c** Glucose-induced insulin secretion from adult human islets. All values are shown as filled triangels, the bars indicate means ± SEM. **d** Oscillatory activity of an islet evoked by 10 mM glucose. The islet was within a pancreatic slice placed on a MEA. **e** Effect of TTX application on oscillatory activity of a human islet in the presence of 10 mM glucose and 100 µM tolbutamide. **f** Lack of effect of TTX on burst activity of a murine islet in the presence of 10 mM glucose.

(250 μM) which hyperpolarized the cells via the opening of K_{ATP} channels. ○ **Fig. 1d** demonstrates that electrical activity can even be obtained from a pancreatic slice of a child with CHI. Burst activity was measured in 10 mM glucose.

Insulin secretion was measured in batches of adult human islets (○ **Fig. 1c**, islets of the same patient as in ○ **Fig. 1a**). The islets secreted only a small percentage of the stored insulin which was less than 0.06 % of 10.0 ± 1.6 μU/10 islets (n = 7) and glucose-induced insulin secretion did not become significant reflecting a low responsiveness of this preparation to glucose.

As reported by Braun et al. [6] gating of voltage-dependent Na^+ channels plays a prominent role in electrical activity of human islets. ○ **Fig. 1e** shows an islet bursting in the presence of 10 mM glucose and tolbutamide (100 μM). This bursting activity was largely and reversibly diminished by the Na^+ channel blocker tetrodotoxin (TTX, 300 nM). Braun and co-workers [6] showed that TTX (300 nM) applied to human islets reduced the AP amplitude by only 25 %, when recorded intracellularly. Furthermore, insulin secretion was reduced by about 50 %, but not abolished [6]. In line with this, we observed bursts of low amplitude in the presence of TTX which we ascribe to Ca^{2+} APs. Measured with extracellular electrodes lower amplitude of the voltage deflections does not necessarily mean that the AP amplitude itself is smaller, but the upstroke of the AP may be much slower matching the differences between voltage-dependent Na^+ (fast upstroke) and L-type Ca^{2+} channels (slow upstroke). In contrast to human islets, TTX did not influence glucose-induced bursts of mouse islets (n = 9) (○ **Fig. 1f**). This is in line with findings by Plant [10] who showed that Na^+ channels of mouse beta-cells are not functional at physiological potentials.

In conclusion, the MEA method emerges as a fast technique for the evaluation of glucose responsiveness of single human islets or islets in tissue slices. Thus, the MEA technique can increase the success rate of experiments in vitro which is very important due to limited access to human biopsies and the low number of islets in a biopsy. As we have already demonstrated the feasibility to cultivate mouse islets for long-term recordings on MEAs [3], this technique will also pave the way for long-term in vitro investigations of human islets.

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